Co-culture of hepatocytes with mesenchymal stem cells for cellular therapy in liver disease

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CO-CULTURE OF HEPATOCYTES WITH MESENCHYMAL STEM CELLS FOR CELLULAR THERAPY IN LIVER DISEASE

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ABSTRACT

A major hurdle facing current hepatocyte transplantation practice is the marginal quality of isolated hepatocytes. Previous studies showed that mesenchymal stem cells (MSCs) could maintain morphology and improve liver-specific metabolism of co-cultured hepatocytes. The present work aimed to optimise the MSCs co-culture system by testing adipose tissue (AT), bone marrow, and umbilical cord-derived MSCs at predefined seeding ratios. Liver-specific metabolism and apoptosis assays were performed to investigate hepatotrophic and antiapoptotic effects of MSCs co-culture. Indirect co-culture was established to investigate the role of paracrine factors in hepatotrophic effect of MSCs co-culture. Hypoxia-preconditioned (HPc) MSCs were co-cultured with hepatocytes to investigate potentiative effect of HPc induction. Intracellular reactive oxygen species (ROS) activity quantitation and antagonisation experiments were performed to investigate whether HPc potentiated MSCs co-culture by an intracellular ROS-dependent mechanism. Tumour necrosis factor alpha (TNF-α), transforming growth factor beta1 (TGF-β1), extracellular collagen, and apoptosis-associated caspase and BAX/BCL-2 signalling pathways were analysed to investigate the contribution of soluble factors, extracellular collagen, and gene signalling to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. All the three types of MSCs exhibited a similar hepatotrophic effect, with a comparable effect even in low-density AT-MSCs co-culture. Hepatotrophic and antiapoptotic effects of MSCs showed a cell contact dependent manner, and HPc potentiated MSCs co-culture by a cell-contact intracellular ROS-dependent mechanism. Decreased hepatocyte autocrine TNF-α, increased MSC autocrine TGF-β1, and enhanced MSCs deposition of extracellular collagen contributed to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction, with downregulated expression of proapoptotic *CASP9*, *BAX*, and *BID* and upregulated expression of antiapoptotic *BCL-2*. It is concluded that synergistic effects of cell contact, intracellular ROS-dependent soluble factors, extracellular matrix, and apoptosis-associated signalling in MSCs co-culture contribute to hepatotrophic effect and HPc-induced potentiative effect. Co-transplantation with MSCs should improve therapeutic effects of HCT by enhancing survival and metabolism of co-transplanted hepatocytes.

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LIST OF ABBREVIATIONS

ACLF	Acute-on-chronic liver failure
ADFs	Adult dermal fibroblast
AFP	Alpha-foetoprotein
ALF	Acute liver failure
ANOVA	Analysis of variance
AT	Adipose tissue
Bax	Bcl-2-associated X protein
Bcl	B-cell lymphoma
Bcl-xL	B-cell lymphoma-extra large
bFGF	Basic fibroblast growth factor
BID	BH3 interacting-domain death agonist
BLK	B lymphoid tyrosine kinase
BM	Bone marrow
BMP	Bone morphogenesis protein
cAMP	Cyclic adenosine monophosphate
Caspases	Cysteine-aspartic proteases
CCK18	Caspase-cleavaged keratin 18
CCL	C-C motif ligand
CCl_4	Carbon tetrachloride
CD	Cluster of differentiation
cDNA	Complement DNA
CK	Cytokeratin 18
CM	Conditioned medium
Ct	Threshold of cycle
Cx	Connexin
CXCR	CXC receptor
CYP450	Cytochrome P450
DCF	2'-7'-dichlorofluorescein
DCFDA	dichloro-dihydrofluorescein diacetate acetyl ester
DCFDH	2',7'-dichlorofluorescin
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
E-cadherin	Calcium-dependent, epithelial-type cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential media
EMI	Epithelial-to-mesenchymal interaction
EMT	Epithelial-to-mesenchymal transition
ES cells	Embryonic stem cells
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate
FLD	Fatty liver disease
FSC	Forward scatter
HBSS	Hank's balanced salt solution
HCl	Hydrogen chloride
HCT	Hepatocyte transplantation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HLR-DR	Human leucocyte antigen D receptor
HOX	Homeobox
HPc	Hypoxic preconditioning/hypoxia-preconditioned
HSC	Haematopoietic stem cells
IGF	Insulin-like growth factor
IL	Interleukin
iPS cells	Induced pluripotent stem cells
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
LSD	Least significant difference

MaIBA	N-(methylamino)isobutyric acid
MAPK/ERK	mitogen-activated protein kinase/Extracellular signal-regulated kinase
MELD	Model for End-Stage Liver Disease
MMP	Metalloproteinase
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
NF	Nuclear factor
NPC	Nonparenchymal cell
OD	Optical density
OLT	Orthotopic liver transplantation
P	Passage
p53	Protein 53
PCR	Polymerase-chained reaction
PI3-K	Phosphatidylinositide 3-kinases
PSR	Picro-sirius red
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Real-time
SD	Standard deviation
SDF	Stromal-derived factor
SLR	Single-lens reflex
SAPK	Stress-activated protein kinase
SRB	Sulforhodamine B
SSC	Side scatter
TGF	Transforming growth factor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
UC	Umbilical cord
VEGF	Vascular endothelial growth factor

WEM	William’s E medium
XIAP	X-linked inhibitor of apoptosis protein

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CHAPTER 1 GENERAL INTRODCUTION

1.1 Overview of Hepatocyte Transplantation

Orthotopic liver transplantation (OLT) is the replacement of a patient's diseased liver with a healthy donor's liver allograft. OLT has been used as the curative modality in acute or chronic conditions, especially inborn liver-based metabolic diseases (Dhawan *et al.*, 2006), that result in irreversible liver dysfunction. The operative technique of OLT has been well established over the last five decades, and patient survival following OLT has been continuously improved due to refinements in surgical care and better knowledge of transplantation immunology (Desai *et al.*, 2008; Beinhardt *et al.*, 2013). The primary limitation of OLT in current transplantation practice is that the number of donor livers available is unfortunately far less than that of recipients on the waiting list (Lo *et al.*, 2004). The operative procedure is also subject to some surgical morbidities and mortalities for both donors in case of living donors and recipients (Ammori *et al.*, 2008). Additionally, patients receiving OLT normally require a life-long immunosuppressive regimen, which impairs patients' quality of life physiologically and psychologically, and places a huge burden on public healthcare system (Schoening *et al.*, 2013).

Hepatocyte transplantation (HCT) has been emerging as a promising alternative treatment modality to OLT for patients who have no access to donor liver or cannot tolerate OLT (Dhawan *et al.*, 2010). The concept of HCT comes from the fact that only a very small portion (approximately 5–10%) of hepatocytes can perform a series of metabolic functions to sufficiently maintain a human subject (Kawashita *et al.*, 2005) In this therapeutic technique, hepatocytes are isolated and purified from donor liver segments that are unused or rejected for OLT mostly due to underlying steatosis (Sagias *et al.*, 2010), using an enzyme perfusion and digestion system. The quality of isolated hepatocytes is subsequently assessed *in vitro* with regards to cell yield, viability, and microbiological safety (Lehec *et al.*, 2009). The preferred recipient site is the liver, into which hepatocytes are delivered through an intra-portal vein catheter (Figure 1.1; Hughes *et al.*, 2012), whilst some alternative sites are also available, including the spleen, pancreas, peritoneal cavity, and subrenal capsule (Hughes *et al.*, 2012). Preclinical studies of HCT on liver disease animal models showed a favourable outcome and led to the clinical use of HCT (Mazaris *et al.*, 2005; Fisher and Strom, 2006).

HCT has been showing a series of clinical benefits in current practice (Table 1.1). The primary advantage of HCT is the minimal invasiveness as compared to OLT (Meyburg *et al.*, 2009). For HCT, isolated hepatocytes are delivered into the liver or other ectopic sites through a catheter using an interventional radiology technique or Doppler ultrasonographic monitoring. This minimally invasive access, thus, minimizes the procedural risks, and offers patients, who cannot tolerate OLT due to poor liver function reserve or pre-existing comorbidities, an additional therapeutic opportunity. HCT is technically less complicated, and this technique can be performed in the setting

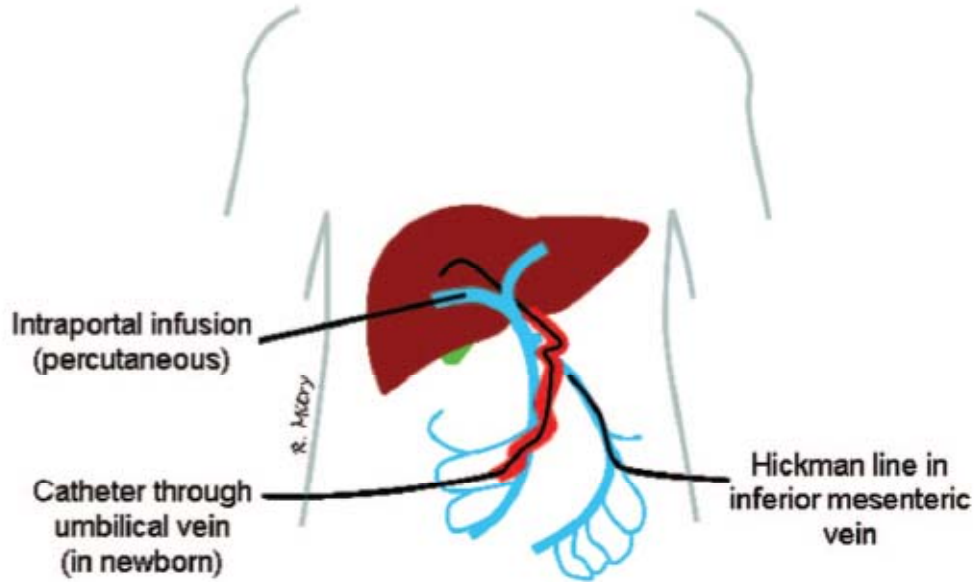

Figure 1.1 **Routes of cell administration for hepatocyte transplantation:** through portal, inferior mesenteric and umbilical veins (Hughes *et al.*, Transplantation, 2012).

Table 1.1 **Clinical benefits of HCT as compared to OLT**

HCT	OLT
• Minimally invasive	• Excessively invasive
• Technically simple	• Technically complicated
• Repeatable if required	• Not repeatable in most cases
• Low medical cost	• Huge financial burden
• One donor liver can be shared by multiple adult or pediatric recipients	• One donor liver can be given to a single recipient only, or an adult and a paediatric recipient
• Preservable and can be used in case of emergency	• Not preservable, and usually not available for emergency use
• Native liver preserved as backup	• Native liver lost for ever
• Not requiring immunosuppression for the treatment of acute liver failure	• Normally requiring life-long immunosuppression

of day surgery. HCT can be repeated within a relative short period if required (Sauer *et al.*, 2012), and the medical costs of HCT is much lower than that of OLT. Hepatocytes isolated from a single donor can be transplanted into multiple recipients, especially in paediatric patients (Mitry *et al.*, 2004). Hepatocytes also can be cryopreserved in cell banks, allowing the immediate accessibility of hepatocytes for emergency transplantation (Fuller *et al.*, 2013). Moreover, the native liver is preserved in place in patients undergoing HCT, which offers a possibility of gene therapy if this becomes clinically feasible in the future. Autologous hepatocytes could be isolated and genetically modified *ex vivo* and back-transplanted to correct metabolic liver disorders (Nguyen *et al.*, 2009).

The use of cell autografts avoids the requirement for a life-long immunosuppressive regimen following HCT, although hepatocytes are presumed to be less immunogenic than the whole liver (Bumgardner *et al.*, 1998). All the aforementioned advantages have been encouraging a wider use of HCT worldwide (Hughes *et al.*, 2012).

1.1.1 *HCT for inborn liver-based metabolic errors*

HCT was initially used for the treatment of inborn liver-based metabolic errors in urea cycle defects, severe unconjugated hyperbilirubinemia (Crigler–Najjar syndrome type 1), factor VII deficiency (haemophilia A), and familial hypercholesterolemia (Dhawan *et al.*, 2006). The outcomes of HCT were most encouraging in patients with inborn liver-based metabolic disorders, as HCT could offer these patients a definite therapeutic benefit (Ribes-Koninckx *et al.*, 2012). In these cases, only a relatively small number of transplanted hepatocytes may be required to compensate for the inherited deficiency of a single liver enzyme, especially in children. In our centre, over ten paediatric patients underwent HCT due to liver-based metabolic disorders, the majority of whom exhibited a clinical improvement without any procedural complications (Dhawan *et al.*, 2005).

1.1.2 *HCT for acute liver failure*

The indication of HCT was further extended to acute liver failure (ALF) as an auxiliary treatment regimen. ALF occurs in severe liver injury with the loss of 80–90% of liver cells within a short period. The prognosis of ALF is highly variable, depending mainly on the underlying etiology. The mortality rate of ALF was up to 80%, but it has been decreasing due to improvements in multidisciplinary intensive care and the advent of emergency liver transplantation (Karvellas *et al.*, 2009). The clinical use of HCT in ALF aims to bridge patients to subsequent OLT, or extend the survival long enough for the native liver to recover and regenerate (Bilir *et al.*, 2000). As the transplanted cells are expected to compensate for the entire liver, the number of hepatocytes required for ALF is normally higher than that for liver-based metabolic disorders, and the infusion of hepatocytes needs to be repeated. Moreover, toxic substances accumulating in ALF patients may be potentially cytotoxic to the transplanted hepatocytes (Mitry *et al.*, 2009). A previous clinical study demonstrated that HCT improved liver function measures in ALF patients, but the overall survival outcome was highly variable (Baccarani *et al.*, 2005). Up to now, it is not possible to draw any conclusion on the overall efficacy of HCT in ALF patients as no controlled trials have been performed.

1.1.3 *HCT for acute-on-chronic liver failure*

HCT has also been attempted in patients with decompensated liver cirrhosis, namely, acute-on-chronic liver failure (ACLF), and aims to prolong a patient's survival and improve one's quality of life, with the hope that OLT will become available at a later time (Kobayashi *et al.*, Transplant Proc, 2000). HCT can improve liver function and overall survival in animal models with chemically-

induced liver cirrhosis (Kobayashi *et al.*, 2000). However, the treatment outcomes from published clinical studies were even more variable for cirrhotic patients (Pareja *et al.*, 2010; Pareja *et al.*, 2013), probably due to the presence of underlying liver fibrosis. The fibrotic lobules prevent the transplanted hepatocytes from passing through the sinusoidal barrier and engrafting into the liver (Gandillet *et al.*, 2005).

1.2 Limitations and Modifications of Hepatocyte Transplantation

It is inevitable that HCT is subject to some technical limitations in terms of accessibility, effectiveness, and safety (Hughes *et al.*, 2012). As compared to OLT, HCT even has a more limited supply of donor liver as hepatocytes used for cell replacement therapy are usually isolated from liver segments unused or rejected for OLT. Another drawback for HCT is the marginal quality of hepatocytes isolated from donor livers unsuitable for OLT mainly due to underlying liver steatosis (Sagias *et al.*, 2010). Steatotic hepatocytes are vulnerable to enzymatic digestion, and processing of fatty liver tissue normally shows a low cell yield and a poor cell viability rate. Therefore, a larger number of hepatocytes of marginal quality are required to maintain normal liver function. Moreover, adult-derived hepatocytes cannot divide or survive long *in vitro* and have a weak repopulation potential, unless the cells are stimulated by appropriate growth factors (Amano *et al.*, 2011). Hepatocytes become dedifferentiated following isolation, and dedifferentiated hepatocytes have an impaired metabolic function (Ambrosino *et al.*, 2005). Hepatocyte transplants are likely to be eliminated by the innate and adaptive immune systems within 7 to 10 days (Han *et al.*, 2009). In animal studies, up to 70% of hepatocytes are primarily cleared by Kupffer cells within the first 24 hours of transplantation, irrespective of synergic or allogeneic grafting (Krohn *et al.*, 2009). Post-transplantation cell loss necessitates the transplantation of a larger number of hepatocytes, in a paradox with the shortage of hepatocyte supply.

1.2.1 *Alternative HCT cell graft sources*

New cell sources have been emerging in recent studies to overcome the primary technical hurdle of HCT, namely, the shortage of donor liver for hepatocyte isolation (Figure 1.2; Fitzpatrick, *et al.*, 2009). Juvenile hepatocytes exhibit a potent repopulation potential as compared to adult cells, but juvenile liver donors are only occasionally available in clinical practice (Walldorf *et al.*, 2004). Hepatocyte xeno-transplantation has been investigated in some animal models showing favorable survival and functional outcomes (Yamamoto *et al.*, 2010); however, this technique is at a high risk of transmitting zoonotic diseases and raises some ethical concerns. Genetically modified or immortalized hepatocytes are reported to have a longer survival by increasing cell replication and reducing cell apoptosis (Tsuruga *et al.*, 2008). The technique of gene manipulation is not clinically available yet and subject to a tumourigenic risk (Trejo-Becerril *et al.*, 2012). Among the newly emerging cell sources, stem cells/progenitor cells are expected to exhibit the most promising outcomes as these cells have been widely investigated for cell replacement therapy. Transplantation of bone marrow (BM) haematopoietic stem cells (HSC) has proved to be clinically effective in the

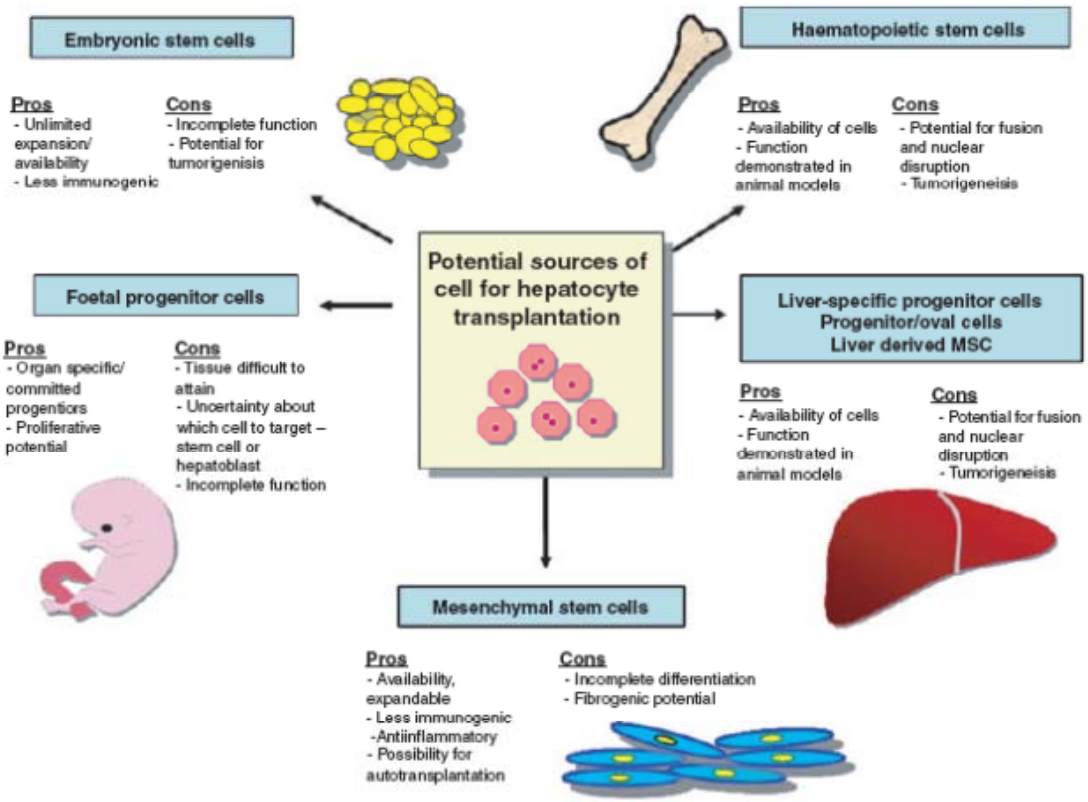

Figure 1.2 Potential alternative sources of hepatocyte for transplantation: ES cells, iPS cells, haematopoietic stem cells, foetal progenitor cells, liver progenitor cells, and MSCs (Fitzpatrick *et al.*, J Intern Med, 2009).

reconstruction of a patient's haematopoietic system. These stem cells can be driven to differentiate into metabolically functional hepatocyte-like cells under specific conditions *in vitro* (Takayama *et al.*, 2012) and promote liver repair and regeneration *in vivo* (Li *et al.*, 2013). The sources of stem cells that have been investigated for HCT include embryonic stem (ES) cells, foetal liver cells, adult-derived hepatic progenitor cells, induced pluripotent stem (iPS) cells, and more popular mesenchymal stem cells (MSCs).

1.2.1.1 ES cells

ES cells are stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo, 4–5 days post-fertilisation in humans. ES cells are believed to be pluripotent rather than totipoent in terms of cell potency (Denker, 2008), and offer a rich source for cell replacement therapy including HCT. ES cells have been successfully induced to differentiate into hepatocytes by way of embryoid body formation in two- or three-dimensional culture *in vitro* (Touboul *et al.*, 2010; Subramanian *et al.*, 2013). Hepatocytic commitment requires the presence of retinoic acid, hepatocyte growth factor (HGF), and β-nerve growth factor (Kuai *et al.*, 2003). These chemically-defined conditions are thought to recapitulate liver development in embryogenesis (Touboul *et al.*, 2010). The immunophenotype and functional activities of ES cells-derived hepatocytes were reported to be identical to those of freshly isolated primary hepatocytes following orthotopic transplantation into the liver or ectopic transplantation into the spleen in animals (Rosen *et al.*, 2003). However, the

harvest of ES cells raises ethical issues as it results in the destruction of fertilised human embryos (de Wert G *et al.*, 2003). The risk of neoplastic transformation in undifferentiated ES cells may be underestimated in current studies, which report an inconsistent presence of teratomas (Choo *et al.*, 2008). Therefore, undifferentiated ES cells should be eliminated prior to transplantation, which is technically difficult in clinical practice.

1.2.1.2 Foetal liver cells

Foetal liver cells are believed to be enriched with hepatoblasts, the progenitor of hepatocytes and cholangiocytes (Masson *et al.*, 2006). These progenitor cells decline rapidly in number after birth and become almost undetectable in adult livers (Schmelzer *et al.*, 2006). Pilot studies on *in utero* transplantation of foetal livers showed a favourable outcome, with advantages in immune tolerance and optimal environment for donor cells in host foetuses (Rosen *et al.*, 2003). The isolation and characterisation of hepatoblasts vary among studies, mainly using liver-specific markers, such as alpha-foetoprotein (AFP), and epithelial cell markers, such as cytokeratins (CK)-18 and -19 (Dan *et al.*, 2006). A number of studies have documented the effectiveness and safety of foetal liver cells in repopulating normal or experimentally injured livers as these cells are destined to mature primarily into hepatocytes (Machimoto *et al.*, 2007). The use of foetal liver cells is also limited by the shortage of donor foetuses and significant ethical concerns (Mychaliska *et al.*, 1998). Moreover, in contrast to expectations, a rodent transplantation study showed that foetal liver cells had a poor liver engraftment and lower repopulation capacity than adult-derived hepatocytes (Haridass *et al.*, 2009).

1.2.1.3 Liver progenitor cells

Liver is well known for its regeneration capacity in response to detrimental factors. As little as one quarter of liver remnant can generate a whole liver *de novo* in healthy subjects (Ju *et al.*, 2012). Liver regeneration has been well described since the age of the original Prometheus myth. This phenomenon is predominately attributed to the quiescent G_0-phase hepatocytes that re-enter the cell cycle, bypass the G_0/G_1 checkpoint, and finally complete mitosis (Satyanarayana *et al.*, 2004). It is a compensatory growth rather than true regeneration of the liver. However, there is some evidence that damaged hepatocytes can be replaced by some liver progenitor cells in adults (Pintilie *et al.*, 2010). These cells are termed as hepatic oval cells in rodents and hepatic progenitor cells in humans. These progenitor cells reside in the canals of Hering, which are located in the periportal region and account for a very small percentage (0.3–0.7%) of liver mass. Adult hepatic progenitor cells are reported to resemble foetal hepatoblasts in terms of phenotype and biopotency (Nava *et al.*, 2005). The identification of oval cells in rats involves multiple immunomarkers, such as oval antigen 6, CK-7, CK-19, and albumin (Terrace *et al.*, 2007). Oval cells can be effectively propagated *in vitro* and directed to the commitment of hepatocytes (Yasui *et al.*, 1997). In animal models of liver injury, oval cells can expand to compensate for the increased turnover of damaged mature hepatocytes when normal G_0-phase hepatocyte mitosis is blocked or in replicative senescence (Yang *et al.*, 2004). However, oval cells are also less accessible for clinical use, and the large-scale expansion of oval cells *in vitro* proves to be technically challenging and subject to loss of potency.

1.2.1.4 iPS cells

iPS cells are a type of artificially programmed pluripotent stem cells that are derived from somatic cells by inducing a forced expression of transcriptional factors. iPS cells were first produced from mouse-derived fibroblasts in 2006 (Takahashi *et al.*, 2006) and from human fibroblasts in 2007 (Takahashi *et al.*, 2007). Theoretically, iPS cells can be reprogrammed to differentiate into any mature cells of ectodermal, mesodermal, and endodermal origin. This technique avoids ethically controversial use of embryos for harvest of ES cells, and also allows production of iPS cells from a patient's own somatic cells, which require no conventional immunosuppression like that following allogeneic transplantation. Functional hepatocytes have been produced from mouse iPS cells that are sequentially subjected to inducing factors (Figure 1.3; Li *et al.*, 2010). These iPS cell-derived hepatocytes share identical morphological and metabolic identities with those derived from ES cells. Human liver disease-specific iPS cells have been available, and these cells can express hepatocyte-specific markers and exhibit a comparable metabolic functionality (Ghodsizadeh *et al.*, 2010). The therapeutic potential of iPS cell-derived hepatocyte-like cells has been justified in a mouse model of carbon tetrachloride (CCl_4)-induced liver injury (Asgari *et al.*, 2011). Primary hepatocytes can also be reprogrammed to iPS cells (Liu *et al.*, 2010). It is interesting that these iPS cells can be directed to differentiate into hepatic progenitor cells as well as mature hepatocytes.

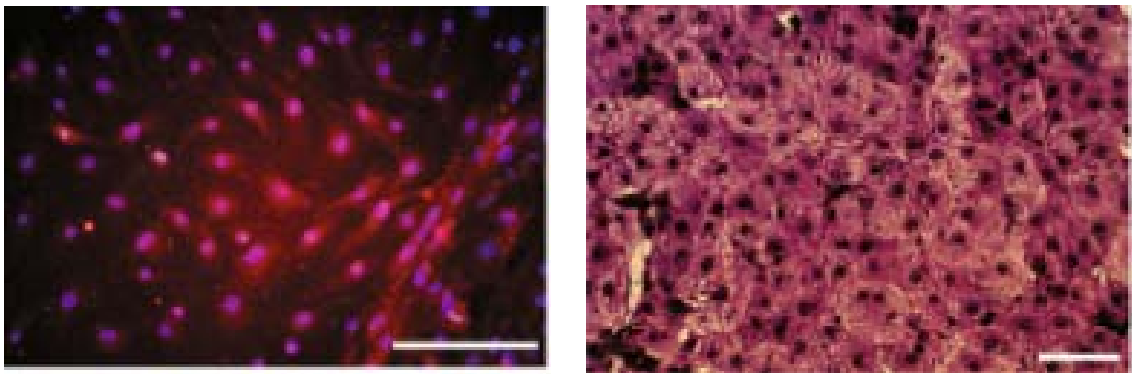

Figure 1.3 Hepatocyte-like cells differentiated from human iPS cells on albumin immunofluorescence microscopy (left panel) and periodic acid-Schiff staining (right panel) (Li *et al.*, J Cell Physiol, 2010).

The use of iPS cells for HCT is faced with some technical challenges with respect to effectiveness and safety. Firstly, the reprogramming of iPS cells shows a very low efficiency; less than 1% of somatic cells can be induced into iPS cells *in vitro*. These techniques usually require precise but clinically less feasible laboratory manipulation. Genomic insertion of transcription factors impairs the safety of iPS cells for cellular therapy as oncogenes are used as reprogramming factors to genomically disintegrate iPS cells and lead to tumourigenesis, a primary safety concern of using iPS cells. Therefore, it is critical to purify differentiated cells by eliminating undifferentiated iPS cells prior to clinical transplantation. Fluorescence activated cell sorting is the preferred method; however, hepatocytes are known to have a complex immunophenotypic profile, and cell sorting may miss a large number of less differentiated progenitor cells with a huge potential of hepatic

regeneration. It remains controversial whether iPS cells are functionally equivalent to ES cells (Bilic and Izpisua, 2012; Puri and Nagy, 2012). It has been reported that iPS cells have a lower efficiency than ES cells in terms of hepatocytic differentiation (Li *et al.*, 2010; Jozefczuk *et al.*, 2011). Moreover, iPS cell-derived hepatocytes were reported to express high-level AFP but low-level albumin, urea, and CYP450, as compared to primary human hepatocytes (Yu *et al.*, 2012). This finding suggests that iPS cell-derived hepatocytes are not completely mature with respect to genotype and phenotype.

1.2.1.5 Haematopoietic stem cells

Haematopoietic stem cells (HSCs) are multipotent progenitor cells that differentiate into myeloid and lymphoid lineages. HSCs transplantation has been well established in the last four decades for treating haematological and autoimmune disorders. HSCs share a stem cell marker Thy-1 with hepatic oval cells, the liver progenitor cells in rats (Petersen *et al.*, 1998). An *in vitro* study confirmed that CD45+ HSCs could be driven into hepatogenesis in the presence of HGF (Zhao *et al.*, 2003). However, it remains controversial whether HSCs can differentiate into hepatocyte-like cells or become fused with hepatocytes *in vivo*. Sex-mismatched peripheral blood HSCs transplantation studies showed that hematopoietic donor chimera cells expressing hepatocyte markers were present in the liver as early as two weeks after transplantation, suggesting the possible differentiation of circulating HSCs into mature hepatocytes (Körbling *et al.*, 2002; Mirzania *et al.*, 2010). Camargo *et al.* (2004) reported that functional hepatocytes derived from HSCs were primarily mature myelomonocytic cells spontaneously fusing with host hepatocytes. It is a technical challenge to induce hepatocytic differentiation of HSCs which requires manipulation in culture over a long period (Miyazaki *et al.*, 2004), although using a combination of multiple growth factors may facilitate HSCs differentiation into hepatocytes (Sellamuthu *et al.*, 2011). The therapeutic role of HSCs in liver disease needs to be critically reassessed. Cantz *et al.* (2004) reported that genetically-labelled HSC transplants could not be detected in the liver or other visceral organs of mice undergoing extended major hepatectomy or chemical injury, although these cells were mobilised by granulocyte colony-stimulating factor.

1.2.1.6 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are mesoderm-derived multipotent stem cells that normally differentiate into a variety of mesenchymal-type cells, including osteoblasts, chondrocytes, and adipocytes. Typical human MSCs manifest a fibroblast-like appearance in a vortex shape. No single cell surface marker can differentiate MSCs from other cell lines; however, undifferentiated MSCs are shown to highly express mesenchymal cell markers, such as CD73, CD105, and CD 106, rather than HSC markers, namely, CD34 and CD45. Novel markers have been emerging and are commercially available for the detection and isolation of MSCs from human or animals, such as bone morphogenetic protein (BMP), stem cell factor receptor, and Stro-1 (Ning *et al.*, 2011). Substantial variability exists in the markers defining MSC population among reports, probably due to the intrinsic heterogeneity of MSCs. As with other multipotent stem cells, MSCs have a

substantial capacity for self-renewal while maintaining their multipotency. MSCs have been artificially driven to differentiate into various cell lines, including epidermal cells (Chun-mao *et al.*, 2007), epithelial cells (Liu *et al.*, 2013; Wu *et al.*, 2013), endothelial cells (Katikireddy *et al.*, 2013), islet cells (Gopurappilly *et al.*, 2013; Marappagounder *et al.*, 2013; Zanini *et al.*, 2011), myocytes (Khani *et al.*, 2013), and specific-type neurons (Yang *et al.*, 2013). For hepatic differentiation, three sources of MSCs have been investigated: BM (Soleimani and Nadri, 2009), umbilical cord (UC) blood (Laitinen and Laine, 2007) or matrix (Wang *et al.*, 2004), and adipose tissue (AT; Neupane *et al.*, Tissue Eng Part A, 2008). A number of studies have examined metabolic functions of MSCs-derived hepatocytes and shown a promising therapeutic prospect (Kang *et al.*, 2005; Sgodda *et al.*, 2007; Ishii *et al.*, 2008; Aurich *et al.*, 2009; Piryaei *et al.*, 2011; Liang *et al.*, 2012; Brückner *et al.*, 2013).

BM-derived MSCs MSCs were first reported to be present in BM, namely, BM-derived MSCs, which co-exist with another stem cell line, namely, HSCs. When supplemented with fibroblast growth factor (FGF)-4 and HGF, a large percentage of rat BM-derived MSCs are induced to exhibit a hepatocyte-like morphology *in vitro* (Kang *et al.*, 2005). These hepatocyte-like cells are capable of secreting albumin, synthesising urea, and storing glycogen. The differentiation media have been optimised in multiple *in vitro* studies, but remain FGF-4- and/or HGF-based. The supplementary growth factors and cytokines that favour directed hepatocytic differentiation consist of insulin-like growth factor 1 (IGF-1; Ayatollahi *et al.*, 2011), β-nerve growth factor (Feng *et al.*, 2011), hepatocyte nuclear factor (NF) 4α (Chen *et al.*, 2010), and oncostatin M (Lee *et al.*, 2004). Other gene products, such as AFP (Ishii *et al.*, 2008), alkaline phosphatase (Kosmacheva *et al.*, 2011), and CK-18 (Kang *et al.*, 2005; Lange *et al.*, 2005[a]; Lange *et al.*, 2005[b]; Lange *et al.*, 2006) have also been used to characterise MSCs-derived hepatocytes under specific inductive circumstances.

UC-derived MSCs UC-derived MSCs are concomitantly present in cord blood and matrix (Wharton's jelly). This type of MSCs has been successfully transdifferentiated into hepatocytes using a protocol similar to that for BM-derived MSCs, although with a lower efficiency (Hong *et al.*, 2005). Human UC-derived MSCs were reported to repopulate and engraft into the liver of a cirrhotic rat model (Jung *et al.*, 2009). However, a xenotransplantation experiment showed that regenerated hepatocytes were actually chimera of donor MSCs and recipient hepatocytes; UC-derived MSCs expressed human albumin and Hep par 1, but murine CK-18, following transplantation into the liver of immunodeficient mice (Sharma *et al.*, 2005). It was reported in the same rodent model that human UC-derived MSCs could differentiate into mature hepatocytes in the absence of cell fusion (Newsome *et al.*, 2003). Interestingly, following transplantation into CCl_4-induced fibrotic rat liver, UC-derived MSCs restored liver function by secreting bioactive cytokines and promoting hepatocyte regeneration rather than differentiating into hepatocytes *in vivo* (Tsai *et al.*, 2009). This contradiction may be attributed to intrinsic variations in UC-derived MSCs.

AT-derived MSCs A newly emerging source of MSCs, AT-derived MSCs are readily available and ethically less controversial (Figure 1.4; Banas *et al.*, 2007). Zuk *et al.* (2001) isolated multipotent stem cells, which mimicked BM-derived MSCs in morphology, from human liposuction

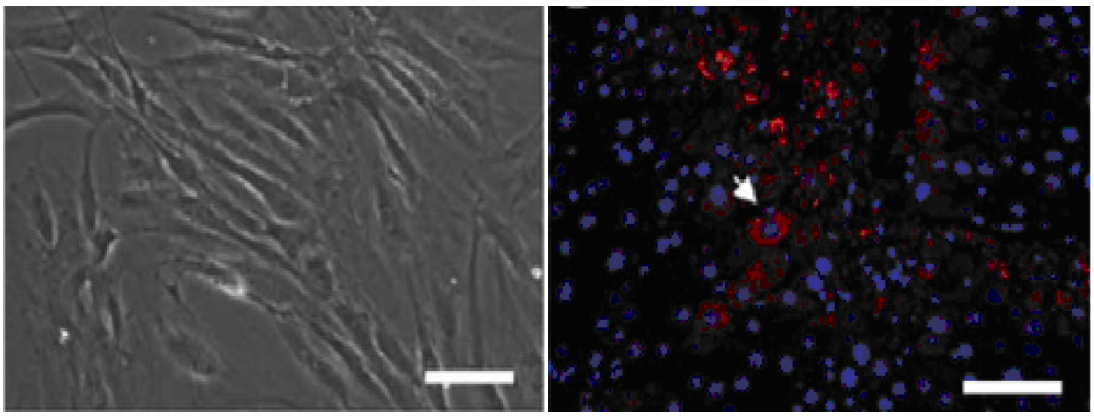

Figure 1.4 Primary human MSCs isolated from AT (left panel) and albumin-immunopositive hepatocyte-like cells (right panel) differentiated from AT-derived MSCs transplanted into CCl4 liver injury model (Banas *et al.*, Hepatology, 2007).

specimens in 2001. The immunophenotypes of AT-derived MSCs are basically similar to those of other sources of MSCs, although almost all AT-derived MSCs express CD49f and CD54, which are rarely expressed in BM-derived MSCs (Zuk *et al.*, 2001). The primary advantages of AT-derived MSCs are extensive source from body adipose, easy harvest by lipoaspiration, and possibility of autologous transplantation. Additionally, AT-derived MSCs are found to be more readily reprogrammed into iPS cells than BM-derived MSCs probably due to the variation in the microenvironment of origin (Mosna *et al.*, 2010). AT-derived MSCs have been driven to differentiate into hepatocyte-like cells with hepatocyte metabolism and hepatocyte-specific transcripts (Ohnishi *et al.*, 2011). Compared to undifferentiated cells, pre-differentiated AT-derived MSCs exhibit a more efficient engraftment into recipient liver (Okura *et al.*, 2010). The therapeutic benefits of AT-derived MSCs have been demonstrated in animal models of inherited liver disease (Okura *et al.*, 2011).

Liver tissue-derived MSCs MSCs can also be isolated from foetal liver tissue (Heidari *et al.*, 2013), with a trilineage differentiation potential identical to BM- and AT-derived MSCs and a proliferative capacity similar to BM-derived MSCs. Human foetal liver-derived MSCs share identical MSCs immunophenotype and potential of multilineage differentiation, and inhibit mitogen-induced lymphocyte proliferation *in vitro* (Götherström *et al.*, 2003). Foetal liver-derived MSCs were reported to be immunosuppressive on T-lymphocytes (Giuliani *et al.*, 2011). These liver-derived MSCs have also been isolated from human adults, basically same to BM-derived MSCs in terms of genome-wide gene expression (Pan *et al.*, 2011). These mesenchymal-like cells co-express hepatic and mesenchymal markers, including albumin, cytochrome P3A4, vimentin, and alpha-smooth muscle actin (Najimi *et al.*, 2007). These cells are preferentially determined to differentiate into hepatocyte-like cells. Following intrahepatic transplantation, the offspring cells are immunopositive for albumin, pre-albumin, and AFP, and show a favourable engraftment potential. A subset of engrafted MSCs survived over the long term *in vivo* and maintained stem cell characteristics (Najimi *et al.*, 2007).

Limitations There are some unavoidable limitations in using MSCs as cellular source for HCT. Harvest of MSCs is not always possible from the patients themselves for autologous transplantation

or from the donors for allogeneic transplantation. Aspiration of bone marrow is invasive for the donors, and the amount of bone marrow collected from an individual donor is very limited even in a healthy subject. In a diseased or ageing subject, the propagation potential of BM-derived MSCs becomes compromised (Katsara *et al.*, 2011). Use of UC-MSCs requires a long-time preservation, whilst the potency of long-preserved UC-MSCs remains questionable. Whereas AT is enriched with highly proliferative MSCs, it is essential to purify the heterogeneous AT-derived cell population (Zhang *et al.*, 2011). As AT-derived MSCs emerged in the last decade, it was less known whether AT-derived MSCs could differentiated into hepatocytes as effectively as BM-derived and UC-derived counterparts, although a previous study reported a similar hepatogenic differentiation potential and efficiency between AT- and BM-derived MSCs (Taléns-Visconti *et al.*, 2006). It usually takes two to three weeks to drive MSCs differentiation into functional hepatocytes *in vitro*; therefore, it makes MSCs-based HCT less suitable for emergency cases, such as for ALF patients. Functional patterns of differentiated hepatocytes are mainly characterised with albumin secretion, urea synthesis, and glycogen storage as well as CYP450 activity, often at low levels, whereas hepatocytes have complex biochemical activities. It remains an open question whether MSCs can differentiate into other cell lineages other than hepatocytes even in directed conditions. These side products may be detrimental for transplanted hepatocyte or even the recipient. MSCs may be a double-edged sword in liver regeneration due to their pro-fibrogenic potential especially in chronic liver injury (di Bonzo *et al.*, 2008). As with other stem cells, the tumourigenic risk of MSCs cannot be underestimated for HCT. It has been recently discovered that MSCs can promote mammary tumourigenesis and progression by enhancing vascularisation in the microenvironment (Ke *et al.*, 2013) although the role of MSCs in breast cancer remains controversial (Usha *et al.*, 2013).

1.2.2 *Preconditioning of hepatocytes*

1.2.2.1 Supportive factors of hepatocytes in vivo

In a native liver, hepatocytes are supported by hepatocytes themselves, extracellular matrix (ECM), and nonparenchymal cells (NPCs). Hepatocyte-to-hepatocyte contact is believed to be crucial for the maintenance of hepatocyte polarity, morphology, and functionality. Calcium-dependent, epithelial-type cadherin (E-cadherin), a major component of intercellular tight junction, maintains hepatocyte spheroid formation and prevents primary hepatocytes from apoptosis by a caspase-independent mechanism (Luebke-Wheeler *et al.*, 2009). E-cadherin expression is modulated by HGF/MET signalling pathway in multiple carcinoma cell lines, including breast cancer, gastric cancer (Han *et al.*, 2005), pancreatic cancer (Paciucci *et al.*, 1998), bladder cancer, prostate cancer, ovarian cancer, melanoma, hypopharyngeal cancer, and nasopharyngeal cancer.

In tissue engineering and regenerative medicine research, seed cells can be preconditioned and modified by supplementing extrinsic growth factors, trophic cytokines, and ECM, or by manipulating intrinsic gene expression prior to transplantation to improve survival, engraftment, and functionality of transplanted cells (Li *et al.*, 2013). These modifications aim to improve proliferation,

survival, committed differentiation, and biological function of seed cells *in vitro* and *in vivo*. Stem cells are preferred target cells to be modified as these pluripotent cells are capable of self-renewal and maintaining the modified effect in the long-term (Navarro-Alvarez *et al.*, 2009). Among these modifications, HGF modification is frequently used to potentiate the regenerative potential of MSCs for the purpose of cellular replacement therapy (Ido and Tsubouchi, 2009; Ishikawa *et al.*, 2011; Ishikawa *et al.*, 2012; Sun *et al.*, Int J Mol Sci, 2013). Bian *et al.* (2009) reported that HGF-modified MSCs exhibited a greater viability as compared to non-modified counterparts and improved skin graft survival. Chen *et al.* (2011) successfully transplanted HGF-modified UC-derived MSCs to ameliorate ischaemia/reperfusion-induced acute kidney injury via anti-apoptotic and anti-inflammatory mechanisms. Alternatively, HGF can also be loaded onto collagen- and fibrin-based biomaterials as artificial ECM to recruit MSCs and promote wound healing (van de Kamp *et al.*, 2013). The concept of modifying the niche rather than the seed cells is of great clinical significance as this technique avoids costly multi-step procedures of isolating, culturing, and transplanting allogeneic stem cells.

1.2.2.2 Growth factors and cytokines

Hepatocytes are a terminally differentiated, mature cell population with a series of highly complex biochemical and metabolic functions (Clayton *et al.*, 2005). Therefore, it is less feasible and more challenging to modify hepatocytes *in vitro*. A large number of growth factors and cytokines, including HGF at the first place (Nakamura *et al.*, 2011; Li *et al.*, 2013; Xu *et al.*, 2013), vascular endothelial growth factor (VEGF; Sturm *et al.*, 2004; Bockhorn *et al.*, 2007), epidermal growth factor (EGF; Natarajan *et al.*, 2007; Liu *et al.*, 2013), insulin-like growth factor (IGF; Sobrevals *et al.*, 2010; Zaouali *et al.*, 2010), transforming growth factor (TGF; Meindl-Beinker *et al.*, 2012), basic fibroblast growth factor (bFGF; Ma *et al.*, 1999), tumour necrosis factor (TNF; Sudo *et al.*, 2008; McMahan *et al.*, 2013), and interleukin (IL) 6 (Sudo *et al.*, 2008; Tiberio *et al.*, 2008), are known to exhibit a marked *in vitro* and *in vivo* hepatocytotrophic effect with respect to hepatocyte survival and regeneration in animals and humans. Microprinted array is a more efficient technique for delivering growth factors to hepatocyte culture on the standard collagen monolayer. Printed arrays of HGF and BMP-7 exhibit antifibrotic and antiapoptotic effects on primary rate hepatocytes (Jones *et al.*, 2010). However, it is not cost-effective to supplement hepatocytotrophic growth factors or cytokines directly into culture medium or surface of hepatocytes as trophic effect of soluble factors can only remain for several days. Additionally, hepatocytotrophic factor supplementation cannot be repeated once hepatocytes have been transplanted.

1.2.2.3 Gene transfection

Gene transfection seems to be a more effective technique for hepatocyte modification. This technique allows the delivery of target genes into donor hepatocytes to overexpress therapeutically efficacious proteins even though the donor cells may be of marginal quality. Additionally, autologous recipient hepatocytes can also be treated as donor cells using the gene fusion or transfection technique to compensate or even correct underlying liver diseases, especially for metabolism-based

disorders. Gene therapy has been experimentally attempted at the levels of both hepatic progenitor cells and mature hepatocytes. Li *et al.* (2012) simultaneously transplanted HGF-modified hepatic oval cells together with orthotopic liver transplantation, and this modification decreased liver allograft rejection and prolonged graft survival in rat recipients. Ajioka *et al.* (2001) co-transplanted hepatocytes transfected with multiple hepatotrophic genes, including HGF, TNF, and VEGF, to establish a heterotropic liver tissue mass with a favourable access to the blood circulation. More encouragingly, Wu *et al.* (2010) transfected human coagulation factor IX into mouse hepatocytes and transplanted gene-modified cells intrasplenically into factor IX knockout mice, a haemophilia B model. Genetically engineered hepatocytes exhibited a significantly higher plasma factor IX clotting activity than the wild-type counterpart with a similar engraftment and factor IX production efficiency. However, the use of genetically modified hepatocytes is inevitably subject to technical limitations and safety concerns although all the experimental studies had shown promising results. It usually takes a few days to complete conventional hepatocyte gene transfection in primary culture. Such a delay prior to HCT is not suitable for clinical use as patients, especially those suffering from ALF, are in urgent need of HCT. Kuge *et al.* (2006) successfully reduced infection time down to only one hour using an adenoviral vector-based transfection system but at the risk of uncontrollable viral infection in the recipient. Viral vector transduced hepatocytes also require the host immune tolerance to maintain long-term transgene expression (Puppi *et al.*, 2004), although mature hepatocytes are known to be relatively less immunogenic *in vivo* (Bumgardner *et al.*, 1998). Moreover, overexpression of hepatocyte proliferation-associated genes, such as HGF, may lead to carcinogenesis and dissemination in the recipient. Upregulated HGF signalling is historically known to drive the occurrence (Yamagamim *et al.*, 2002), progression (Jia *et al.*, 2013), metastasis (Chau *et al.*,2008), recurrence (Mizuguchi *et al.*, 2009), metastasis (Ogunwobi *et al.*, 2013) and chemoresistance (Lasagna *et al.*, 2006; Yu *et al.*, 2013) of hepatocellular carcinoma, as well as liver-metastatic colorectal cancer (Min *et al.*, 2012).

1.2.2.4 ECM

ECM supportive effect is known to be essential for maintaining physiological hepatocyte morphology, survival, proliferation, differentiation, and liver-specific function, especially in the sense of the long-term *in vitro* culture. ECM modulates hepatocyte survival (Giri *et al.*, 2003; Pinkse *et al.*, 2004; Ohashi *et al.*, 2005; Zavan *et al.*, 2005), viability, morphology (Zavan *et al.*, 2005), phenotype (Page *et al.*, 2007; Woodrow *et al.*, 2009), proliferation (Hammond *et al.*, 2011), differentiation (Sidhu *et al.*, 2004; Kimata *et al.*, 2006; Kimata *et al.*, 2006), maturation (Brill *et al.*, 2002), metabolism (Depreter *et al.*, 2000; Sellaro *et al.*, 2010), repair (Ma *et al.*, 1999), and regeneration (Hammond *et al.*, 2011), via paracrine factors, such as HGF (Schuppan *et al.*, 1998), hepatocyte NF 4 (Oda *et al.*, 1995), bFGF (Ma *et al.*, 1999), and cell adhesion molecules, such as integrin (Pinkse *et al.*, 2004).

Multiple novel tissue engineering biomaterials, namely, scaffolds, have been investigated to construct the optimal residential environment for hepatocytes *in vitro*. The scaffolds are usually composed of naturally hyrdrogels, such as chitosan (Elçin *et al.*, 1998) and alginate (Elkayam *et al.*,

2006), or artificially synthesised high-molecule glycopolymers, such as polyethylene glycol (Underhill *et al.*, 2007) and polycaprolactone/polylactic-co-glycolic acid (Shim *et al.*, 2013). ECM modification is even more beneficial for maintaining long-term hepatocyte culture *in vitro* for the purpose of drug hepatotoxicity screening (De Bruyn *et al.*, 2013), bioartificial liver support (Kinasiewicz *et al.*, 2008; Giri *et al.*, 2013), and anti-hepatitis virus agent experiments (Molina-Jimenez *et al.*, 2012). These scaffolds can be further surface-modified with major components of ECM, such as collagen (Hou *et al.*, 2011), fibrin (Gwak *et al.*, 2004), laminin (Tai *et al.*, 2010), and fibronectin (Mehta *et al.*, 2010). Common growth factors, such as HGF (Seo *et al.*, 2006; Nelson *et al.*, 2011), VEGF (Kedem *et al.*, 2005; Hou *et al.*, 2011), EGF (Koyama *et al.*, 2009), and IGF (Nelson *et al.*, 2011) can also be incorporated into surface-modified bioscaffolds using the nano-material technology to augment survival and functionality of seeded hepatocytes. The emergence of an injectable bioscaffold allows hepatocyte-scaffold transplants to be delivered using minimal invasive access techniques, especially for cirrhotic patients. Navarro-Alvarez *et al.* (2010) constructed a peptide nanofiber-based, three-dimensional scaffold, loaded with growth factors and seeded with immortalised human hepatocytes, into a tissue-engineered liver graft that could be injected intramuscularly. This engineered liver graft could maintain liver-specific gene expression and functionality *in vitro* to correct acute or chronic liver failure in animal models.

1.2.2.5 Bioscaffold

A potential additive benefit of using growth factor surfaced bioscaffold is that controlled release of growth factors may improve engraftment and survival of hepatocytes by promoting angiogenesis other than offering attachment alone in the long term (Hou *et al.*, 2011). Kedem *et al.* (2005) delivered sustained VEGF to enhance scaffold vascularisation and improved hepatocyte transplant engraftment in the host liver lobe by 4.6 fold up to 12 days following transplantation. However, Smith *et al.* (2006) augmented hepatocyte transplant short- rather than long-term survival by delivering EGF and HGF using a VEGF-designated porous polymer scaffold. These findings suggest that hepatocyte transplant engraftment requires simultaneous delivery of multiple signals. Therefore, a designated scaffold that can deliver multiple growth factors is required for the clinical use of hepatocyte-scaffold transplantation. A decellularised whole liver is supposed to be the ideal scaffold to reconstruct a ‘‘new’’ liver *de novo* (Baptista *et al.*, 2011; Soto-Gutierrez *et al.*, 2011; Zhou *et al.*, 2011; Shirakigawa *et al.*, 2012; Yagi *et al.*, 2013). Wang *et al.* (2013) reported that decellularised liver matrix effectively supported proliferation and differentiation of murine foetal liver progenitors for up to 2 weeks. However, the question from preclinical, experimental studies is how to acquire an ideal decellularised liver scaffold: the use of a healthy donor liver has to sacrifice billions of healthy hepatocytes, while that of a diseased, such as fibrotic, donor liver carries a high risk of fibrogenesis and carcinogenesis due to the unfavourable environmental factors. Combination of human-derived hepatocytes and swine-derived acellular liver scaffold may be a good solution. Barakat *et al.* (2012) established an acellular porcine liver scaffold while preserving the native architecture and most ECM components, which facilitated maturation of human foetal hepatocytes co-cultured with foetal stellate cells into differentiated hepatocytes with respect to immunohistochemistry and biochemical

metabolism. Again using animal-derived material is subject to ethical challenge and risk of zoonosis.

1.3 Heterotypic Interactions of Hepatocytes with Non-hepatocytic cells

Once isolated and cultivated *in vitro*, hepatocytes are deprived of hepatocyte-to-hepatocyte contact, ECM support, and hepatocyte-to-non-hepatocytic cell communication. Hepatocyte mono-culture, if not chemically defined, normally exhibits a marked reduction in phenotype and metabolic functionality, such as a rapid decrease in albumin secretion and AFP expression (Bhatia *et al.*, 1998). This phenotypic and functional regression is attributable to the *in vitro* dedifferentiation of hepatocytes resulting from a complex, wide-ranging change in proteomics and depending on the culture condition (Rowe *et al.*, 2010). Co-cultivation of multiple non-hepatocytic cells, mainly of mesenchymal origin, is reported to maximise hepatocyte function *in vitro*. NIH/3T3 cells, a fibroblast cell line obtained from mouse embryos, are most frequently used as feeder cells in hepatocyte co-culture (Lu *et al.*, 2005). This cell line is known to regulate HGF and MET (HGF receptor) expression, and secrete adhesion molecules (Halverson *et al.*, 1999). Co-culture with NIH/3T3 cells significantly improved albumin secretion (Nishikawa *et al.*, 2008) and CYP450 activity (Chia *et al.*, 2005) in hepatocytes as compared to mono-cultured counterparts in the short term. TGF-β1 was thought to mediate this enhanced liver-specific metabolism as extracellular activation of latent TGF-β1 was upregulated in the co-culture and TGF-β1 neutralisation diminished this functional enhancement (Chia *et al.*, 2005). Moreover, three-dimensional heterotypic co-culture with NIH/3T3 cells can even maintain a high level of albumin secretion and CYP450 activity for almost two weeks, while hepatocyte homo-culture becomes metabolically inactive after the first week (Lu *et al.*, 2005).

Co-culture with NIH/3T3 cells is, however, subject to a high variability in hepatocyte metabolic enhancement, with respect to albumin secretion, urea synthesis, and CYP450 activity (Gregory *et al.*, 2001). This variation can be attributed to oxygen uptake among co-culture systems varying in seeding density (Cho *et al.*, 2007). Moreover, the use of fibroblasts may risk promoting liver fibrosis *in vivo*, if transplanted with hepatocytes, although it has not been reported in current literature regarding HCT. It was reported that TGF-β1-expressing NIH/3T3 cells, if injected intracavernously, could result in rat penile fibrosis (Ryu *et al.*, 2005). Therefore, this technique is more suitable for developing bioartificial liver support systems (Washizu *et al.*, 2001; Seo *et al.*, 2006).

1.3.1.1 Co-culture of hepatocytes with non-parenchymal liver cells

A native liver is composed of parenchymal cells – hepatocytes accounting for 80%, and NPCs, also called stromal cells, for 6.5% of the total liver volume (Kmieć, 2001). Liver NPCs consist of Kupffer cells, sinusoidal endothelial cells, and stellate cells. These cells play a regulatory role in hepatocyte maintenance, proliferation, apoptosis, and maturation (Melgert *et al.*, 2000; Zinchenko *et al.*, 2006; Zhang *et al.*, 2009). Activation of NPCs is critical for hepatocyte regeneration in the well-established 70% partial hepatectomy model (Sakuda *et al.*, 2002). This proliferative subpopulation

of hepatic cells also carries the potential for differentiation into metabolically functional hepatocyte-like cells in the presence of HGF and EGF or FGF-4 (Duret *et al.*, 2007). Co-culture with liver sinusoidal endothelial cells can help maturation of foetal and neonatal hepatocytes and maintain albumin secretion in rats (Morin *et al.*, 1986). Co-culture with NPCs, including sinusoidal endothelial cells, hepatic stellate cells, and partially activated Kupffer cells, can even maintain and enhance liver-specific gene expressions of non-serum-fed hepatocytes in the presence of EGF, bFGF, and hepatocyte conditioned medium (CM; Ries *et al.*, 2000). This *in vitro* co-culture system is expected to sustain the metabolic function of hepatocytes in the long term (Shulman and Nahmias, 2013). In addition to serving as a nourishing feeder for mature hepatocytes, multiple NPCs are reported to direct hepatogenic differentiation of ES cells and MSCs. NPCs co-culture drives mouse ES cells to differentiate into hepatocyte-like cells, at a rate of approximately 70% with respect to albumin production, ammonia metabolism, and drug detoxification, in the presence of HGF and dexamethasone (Soto-Gutiérrez *et al.*, 2007). Hepatic stellate cells are also reported to contribute to hepatocytic differentiation of BM-derived MSCs (Deng *et al.*, 2008). Hepatic stellate cells activated by Kupffer cells exert a modulatory effect on MSCs hepatic differentiation mediated by IL-6 and -10 (Parekkadan *et al.*, 2007).

NPCs exert both positive and negative effects on neighbouring hepatocytes in paracrine, cell-matrix, and cell-cell manners. Activated hepatic stellate cells help co-cultured hepatocytes to aggregate rapidly into well-defined viable spheroids (Thomas *et al.*, 2005) and regulate hepatocyte proliferation *in vitro* (Uyama *et al.*, 2002); these spheroids show a complex ECM support and hepatic ultrastructure (Thomas *et al.*, 2005). Upregulated HGF expression underlies hepatocyte co-culture with hepatic stellate cells, suggesting that the co-culture undergoes a post-traumatic regenerative process (Thomas *et al.*, 2005). Aberrant activities of NPCs are also attributed to liver fibrosis through releasing inflammatory mediators and reactive oxygen species (ROS; Cohen and Nagy, 2011). NPCs are also involved in liver allograft rejection as this cell population is readily targeted by cytotoxic antibodies and complement (Astarcioglu *et al.*, 1995). This beneficial technique has been investigated for bioartificial liver support systems (Nedredal *et al.*, 2007; Soto-Gutierrez *et al.*, 2010) and *in vitro* drug toxicity screening systems (Kostadinova *et al.*, 2013). The application of hepatocytes and NPCs co-culture in HCT is limited by a fact that highly variable NPCs themselves may inevitably result in a variation in co-culture hepatocytotrophic effect. NPCs supportive effect on co-cultured hepatocytes can be present for no more than 7 days in the absence of specific growth factors, such as HGF, EGF (Kan *et al.*, 2004), VEGF, and IL-6 (Kang *et al.*, 2004). Moreover, it is a safety concern that NPCs are historically known to be the major source of collagen production if profibrotic factors are present.

1.3.1.2 Co-culture of hepatocytes with MSCs

Specific interactions between epithelial cells and mesenchymal-derived cells are known to be required for liver morphogenesis (Tanimizu *et al.*, 2007). Heterotypic co-culture with MSCs shows a significantly higher metabolic activity, including albumin secretion, urea synthesis, and CYP450

activity, than homotypical mono-culture, especially after one week of cultivation (Gu *et al.*, 2009[a]; Gu *et al.*, 2009[b]; Gu *et al.*, 2009[c]). BM-derived MSCs are stromal supporting scaffolds for HSC by secreting a series of crucial cytokines and growth factors (Pontikoglou *et al.*, 2011). Hepatocyte multiplication can be stimulated by multiple soluble factors *in vitro*, including IL-6, bFGF, and TGF-α. Similar to NPCs, BM-derived MSCs assist primary hepatocytes in the formation of spheroids in co-culture (Gu *et al.*, 2009[c]). Scanning electron microscopy shows the establishment of cell-matrix and cell-cell contacts of hepatocytes on top of MSCs monolayer, with a well-organised three-dimensional tomography (Figure 1.5; Gu *et al.*, 2009[c]).

Figure 1.5 Hepatocytes co-cultured with BM-derived MSCs (left panel) exhibit a three-dimensional tomography on scanning electron microscopy (right panel) (Gu *et al.*, J Cell Physiol, 2008).

Co-cultured hepatocytes exhibit a lower G_0/G_1-phase fraction, but a higher G_2/S-phase fraction on cell cycle analysis, suggesting the activation of quiescent hepatocytes by BM-derived MSCs in co-culture (Gu *et al.*, 2009[b]). The insertion of a semi-permeable porous membrane between MSCs culture and hepatocyte culture eliminates the cell-cell contact but still increases the hepatocyte function (Gu *et al.*, 2009[c]). This suggests that trophic effect of MSCs co-culture results from soluble cytokines and growth factors released from MSCs. The potential trophic soluble factors consist of HGF, IL-6, and TNF-α as shown by the neutralisation experiments (Gu *et al.*, 2009[a]). Moreover, ECM, such as fibronectin, laminin, and collagen type I/III/V, deposited by MSCs may also contribute to enhanced hepatocyte function in co-culture, as validated in ECM gene knockdown experiments (Gu *et al.*, 2009[c]).

In addition to maintenance and modification of hepatocytes, MSCs co-culture also facilitates hepatic differentiation of stem cells or progenitor cells *in vitro* (Lange *et al.*, 2005[b]; Lange *et al.*, 2006; Qihao *et al.*, 2007). Co-culture with foetal liver-derived MSCs helps hepatic maturation of hepatic progenitor cells and hepatic differentiation of ES cells (Ishii *et al.*, 2010). ES cells-derived hepatocytes are immunocytologically characteristic of mature hepatocytes with a higher metabolic activity (Ishii *et al.*, 2010).

As MSCs are known to be potentially hepatogenic both *in vitro* and *in vivo* (Ji *et al.*, 2012;

Wang *et al.*, 2012) and immunomodulatory (Yi *et al.*, 2012; Zhang *et al.*, 2012), MSCs-based hepatocyte co-culture appears to be an effective modality for improving current HCT practice. It was reported that co-culture with BM-derived stromal cells augmented hepatocyte-specific metabolic functionality up to one month (Mohajerani *et al.*, 2010). Foetal liver-derived MSCs improve engraftment of transplanted hepatocytes (Joshi *et al.*, 2012); hepatocytes detached from BM stromal cell co-cultures also have a better engraftment following the transplantation into the spleen as compared to those detached from fibroblast cell co-cultures (Mohajerani *et al.*, 2010). MSCs co-culture can improve the marginal quality of hepatocytes isolated in current practice and minimise the number of hepatocyte transplants required for a favourable therapeutic outcome, without requiring delicate, costly laboratory manipulation. This technique has been successfully used to modify bioartificial liver system for treating ALF (Yagi *et al.*, 2009; Yang *et al.*, 2013), and it is also potentially promising to improve HCT in the treatment of ALF and liver-based metabolic disorders.

1.3.1.3 Possible Contribution of MSCs to HCT

Co-transplantation of MSCs with islet cells has been reported for the treatment of experimental diabetes mellitus (Sakata *et al.*, 2011). MSCs co-transplants can improve islet graft survival and function *in vitro*, as well as engraftment revascularisation *in vivo* (Ito *et al.*, 2010; Rackham *et al.*, 2011; Kerby *et al.*, 2013). The incorporation of MSCs is also expected to bring some additional benefits to current HCT practice. This technique is likely to help overcome the major hurdles of current HCT practice, namely, shortage of hepatocytes available for transplantation and marginal quality of isolated hepatocytes.

Firstly, MSCs can modify hepatocyte morphology and functionality (Gu *et al.*, 2009[a]; Gu *et al.*, 2009[b]; Gu *et al.*, 2009[c]). It is possible that MSCs co-culture decreases the number of hepatocytes required for a given recipient and gives a similar or even better therapeutic outcome. It has been proposed that co-transplantation of iPS cells-derived hepatocytes and MSCs should be an effective treatment alternative to orthotopic liver transplantation for treating end-stage liver disease (Liu *et al.*, 2009).

Secondly, co-transplanted MSCs can transdifferentiate into hepatocytes following the engraftment. BM-derived MSCs co-cultured with foetal liver cells exhibit a high-efficiency hepatocytic differentiation, similar to cells sequentially subjected to stem cell factor, HGF, EGF, and FGF-4 (Lange *et al.*, 2006). Human UC-derived MSCs differentiated into hepatocyte-like cells, without accelerating capillarisation and venularisation of liver sinusoids, in CCl_4-induced liver fibrosis model (Ren *et al.*, 2010). Shi and his colleagues (2009) used co-encapsulated hepatocytes and MSCs transplantation to successfully increase liver function and survival rate of an ALF rat model, and observed *in vivo* transdifferentiation of MSCs into hepatocyte-like cells with respect to albumin expression.

Thirdly, MSCs are known to be immunomodulatory (Lin *et al.*, 2011), and MSCs-derived hepatocytes are reported to less immunogenic (Al Jumah and Abumaree, 2012; Chen *et al.*, 2013;

Hou *et al.*, 2013). The immunomodulatory effect of MSCs has been applied in the therapeutics of some autoimmune and inflammatory diseases (Fiorina *et al.*, 2009; Ohshima *et al.*, 2012), including graft-versus-host disease (Chen *et al.*, 2012; Gregoire-Gauthier *et al.*, 2012; Xia *et al.*, 2012) and liver transplantation (Wan *et al.*, 2008; Popp *et al.*, 2009; Zhang *et al.*, 2012), although it remains controversial (Zhang *et al.*, 2009). Hepatocyte-like cells, which derive from UC-derived MSCs in the presence of HGF and bFGF, neither express major histocompatibility complex II antigen nor induce lymphocyte proliferation *in vitro* (Zhao *et al.*, 2009). The immunomodulatory property of MSCs co-transplanted with hepatocytes may result in less requirement of immunosuppressive agents, as compared to that in conventional HCT, if used for the treatment of inborn liver-based metabolic disorders (Burlina, 2004).

Lastly, trophic factors of MSCs are also expected to be protective for hepatocytes subjected to a detrimental environment, such as in ACLF (Shi *et al.*, 2011), chemically-induced liver injury (Jung *et al.*, 2013; Salomone *et al.*, 2013; Shao *et al.*, 2013; Xagorari *et al.*, 2013), and liver ischaemia-reperfusion injury (Pan *et al*., 2012; Jin *et al.*, 2013). Culture with ACLF serum, which contained high-level TNF-α but minimal EGF and VEGF, caused a high cell detachment rate, low viability, and reduced liver-specific function in human hepatocyte mono-culture; however, MSCs protected co-cultured hepatocytes from ACLF-induced cytotoxicity (Shi *et al.*, 2011). MSCs-conditioned medium can attenuate CCl_4-induced early apoptosis of hepatocytes by IL-6 and fibroblast-like protein 1 signalling (Xagorari *et al.*, 2013). Moreover, MSCs transplantation can ameliorate ROS-induced rat liver ischaemia-reperfusion injury by downregulating mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) signalling pathway (Pan *et al.*, 2012). However, a randomised, controlled study demonstrated that autologous bone marrow MSC transplantation had no therapeutic benefit for patients with decompensated cirrhosis with respect to absolute changes in Child-Pugh classification, the Model for End-Stage Liver Disease (MELD) score, serum albumin, international normalised ratio, serum transaminases, and liver volume (Mohamadnejad *et al.*, 2013). Moreover, the profibrotic effect of co-transplanted MSCs cannot be ignored as it remains controversial whether MSCs are antifibrotic or profibrotic following intraportal transplantation (Rabani *et al.*, 2010; di Bonzo *et al.*, 2008).

In summary, HCT is an effective treatment alternative to OLT for ALF and inborn liver-based metabolic disorders. However, current HCT practice is subject to two major hurdles, namely, shortage of donor livers and marginal quality of isolated hepatocytes. Alternative cellular sources, including ES cells, foetal liver cells, adult-derived hepatic progenitor cells, iPS cells, and MSCs, have been investigated for the preclinical use in HCT. Differentiation of stem/progenitor cells into hepatocyte-like cells requires delicate, costly laboratory manipulation and risks carcinogenesis of undifferentiated cells. Heterotypic interaction with NPCs plays a regulatory role in hepatocyte maintenance, proliferation, apoptosis, and maturation. MSCs co-culture is expected to have multiple contributions to current HCT practice. MSCs can modify hepatocyte phenotype and metabolism; hepatocyte-committed transdifferentiation of MSCs may reduce liver mass required for HCT; immunomodulatory MSCs co-transplants may result in less requirement of immunosuppressive

agents following HCT; and MSCs can also protect co-transplanted hepatocytes from cytotoxicity induced by inflammatory factors or other unfavourable chemical factors, especially in patients with ALF and ACLF. Modification of MSCs and hepatocytes co-culture system is expected to further augment hepatocyte functionality for the clinical use of HCT. Molecular mechanisms underlying MSCs and hepatocytes co-culture are also yet to be delineated for improving current HCT practice.

1.4 Objectives and Hypotheses of PhD Project

MSCs co-culture shows favourable hepatotrophic effect with respect to morphology, cell survival, and liver-specific metabolic functions, as documented in current literature. The present work aimed to modify conventional hepatocyte co-culture with MSCs and further potentiate *in vitro* hepatotrophic effect of MSCs co-culture. Furthermore, it is a primary objective of this project to investigate the molecular mechanisms underlying hepatotrophic and potentiated effects of MSCs co-culture.

1.4.1 *Optimisation of human hepatocyte and mesenchymal stem cell co-culture system in vitro*

Multiple sources of MSCs are available for co-culture with hepatocytes. The first objective of this project is to determine which source of MSCs, namely, AT-, BM-, and UC-MSCs, is the optimal MSCs for hepatocyte co-culture *in vitro*. Additionally, it is investigated in this part of the project whether MSCs co-culture can suppress spontaneous and chemically-induced apoptosis of hepatocytes *in vitro*.

1.4.2 *Hypoxic preconditioning potentiates MSCs co-culture hepatotrophic effect*

As MSCs normally reside in a low-oxygen niche, hypoxic precondition (HPc) is frequently used to optimise MSCs for cellular transplantation use. The second objective of this project is to verify the hypothesis that HPc can potentiate hepatotrophic effect of MSCs. It is also investigated in this part of the project whether HPc-induced potentiative effect depends on intra-MSCs activity of ROS, a pivotal signalling factor in cellular response to oxidative stress.

1.4.3 *Mechanisms underlying hepatotrophic and HPc-induced potentiative effects of MSCs co-culture*

Hepatotrophic effect of MSCs is believed to result from paracrine factors and ECM deposition of MSCs, as well as hepatocyte-to-MSC interaction. The third objective of this project is to investigate whether secretion of TGF-β1 from MSCs and TNF-α from hepatocytes, deposition of extracellular collagen from MSCs, and hepatocyte apoptosis and survival associated signalling pathways are implicated in MSCs co-culture hepatotrophic effect. It is also investigated in this part of the project whether modulatory effects of HPc on soluble factors, ECM, and gene signalling pathways of hepatocytes co-cultured with MSCs depend on intra-MSCs ROS activity.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Sources of MSCs

Primary human AT-MSCs, which were originally extracted from a single human donor's lipoaspirate tissue through mechanical and enzymatic digestion, were purchased from Invitrogen Ltd, Paisley, UK. Primary cultures had been expanded for one passage before cryopreservation. The cell line was immunophenotyped by the supplier as positive for CD29, CD44, CD73, CD90, CD105, and CD166 (>99%), and negative for CD14, CD31, CD45, and Lin1 (< 1%) on flow cytometry.

Primary human BM-MSCs, which were originally extracted from a single human donor's bone marrow through enzymatic digestion, were purchased from Lonza Group, Ltd., Basel, Switzerland. Primary cultures had been expanded for one passage before cryopreservation. The cell line was immunophenotyped by the supplier as positive for CD105, CD166, CD29, and CD44 (>99%), and negative for CD14, CD34, and CD45 (<1%) on flow cytometry.

Primary human UC-MSCs were originally extracted by Dr Yue Wu (Institute of Liver Studies, King's College Hospital NHS Foundation Trust, London, UK) from a single human donor's umbilical cord matrix (Wharton's jelly) through digestion in 1-mg/mL collagenase type I (Sigma-Aldrich, St Louis, MO, USA) as previously reported (Campard *et al.*, Gastroenterology, 2008). The cell line was immunophenotyped as positive for CD13, CD73, CD105, CD90, and CD44 (>99%), but negative for CD31, CD34 and HLR-DR (<1%) on flow cytometry.

The differential potentials of MSCs towards adipocytes, osteoblasts, and chondrocytes, were characterised *in vitro* by the suppliers using specialised differentiation media.

2.2 Subculture of MSCs

The MSC expansion culture media consisted of phenol red-free, low-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen, Paisley, UK), 10% foetal calf serum (FCS; Invitrogen, Paisley, UK), 2-mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), and 100-U/mL penicillin plus 100-µg/mL streptomycin (Invitrogen, Paisley, UK). Cryopreserved cells (approximately 1 million cells per mL and per vial) were quickly swirled and thawed in a 37°C water bath (Grant Instruments, Ltd, Cambridge, UK) for 1 min. The cell suspension was immediately transferred into a 50-mL sterile Falcon® polypropylene conical tube (BD Biosciences, Durham, NC, USA) containing 10-mL pre-warmed culture media and centrifuged at 1,500 rpm (Heraeus Instruments, Newport Pagnell, UK) for 5 min. The cell pellet was subsequently resuspended in pre-warmed culture media, and plated onto a 75-cm^2 tissue culture flask (NUNC A/S, Roskilde, Denmark) at a density of 5,000 cells per cm^2 and at a volume of 10 mL per T75 flask, in a humidified incubator (Heraeus Instruments, Newport Pagnell, UK), in an atmosphere of 95% O_2 and 5% CO_2 and at 37°C. The culture medium was refreshed every 3–4 d.

On day 7 of culture (approximately at 80% confluency), cell cultures were rinsed with phosphate buffered saline (PBS; Invitrogen, Paisley, UK) and detached by adding 2.5-mL 0.25% trypsin/PBS (Invitrogen, Paisley, UK) at 37°C for 5–10 min, and the cell dissociation was stopped by adding 10% FCS/DMEM. The cell suspension was replated and subcultured at a ratio of 1:5 using the same cell culture protocol. The cells were cryopreserved in the culture medium supplemented with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) as multiple aliquots (1 mL per vial) using a freezing container (Thermo Fisher Scientific, Ltd, Loughborough, UK) in a −80°C freezer (New Brunswick Scientific, Cambridge, UK), and the stocks were stored in a −140°C freezer (New Brunswick Scientific, Cambridge, UK). The 6^{th}–8^{th} passages (P6–8) of MSCs were used for experiments. The total number and viability of MSCs for each passage were determined using a Neubauer improved bright-line hematocytometer (Weber Scientific International, Ltd, Hamilton, NJ, USA) and the trypan blue (0.2%; Sigma-Aldrich, St Louis, MO, USA) exclusion technique with a standard inverted light microscope (Leica Microsystems, Milton Keynes, UK) equipped with a digital single lens reflex (SLR) camera (Cannon, Tokyo, Japan).

2.3 Primary Harvest of Human Hepatocytes

The use of human liver tissues was approved by the Research Ethics Committee at King's College Hospital NHS Foundation Trust, London, UK, and conformed the guidelines set out in accordance with *the Human Tissues Act of 2004*. All donors or their legal representatives volunteered to give informed consent in writing for research use.

Primary human hepatocytes were isolated from donor liver tissues unused or rejected for orthotopic liver transplantation using a standard collagenase perfusion technique (Figure 2.1), as previously reported by Mitry (2009). Briefly, the liver tissue was maintained in ice-cold Eagle's minimum essential media (EMEM; Lonza Group, Ltd., Basel, Switzerland) during pre-processing. One or two major patent vessels were cannulated with 16−22G intravenous catheters (Smiths Medical International Ltd., Rossendale, UK) and secured in place using nylon sutures (Tyco Healthcare Group LP, Norwalk, CT, USA). Other minor vascular and biliary vessels on the cut surface were ligated using silk sutures (Ethicon, Inc., Somerville, NJ, USA). The liver tissue was perfused using a MasterFlex® L/S® digital standard drive (Cole-Parmer Instrument Company, London, UK) at a flow rate of 60–80 mL/min. The perfusates contained calcium-free Hank's balanced salt solution (HBSS; Lonza Group, Ltd., Basel, Switzerland), 1-M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich, St Louis, MO, USA), and 0.5-mM ethylene glycol tetraacetic acid (EGTA; Sigma-Aldrich, St Louis, MO, USA), a calcium chelant to disrupt the desmosomal junctions of neighboring hepatocytes. The first perfusion solution was replaced by a second perfusion solution consisting of plain calcium-free HBSS. The post-perfused liver specimens were digested using a third EMEM-based perfusion solution containing 0.05% *Clostridium histolyticum* derived collagenase P (Roche Diagnostics Ltd., West Sussex, UK). The volume of collagenase solution used for the digestion varied with the weight of liver tissue, roughly 250 mL for every 200-gram liver tissue. The collagenase P solution was re-circulated for no more than three

Figure 2.1 Collagenase perfusion system (left panel) and cannulated donor liver segments (right panel) being digested for hepatocyte isolation.

cycles until the specimen was appropriately digested. The buffer solutions were maintained at 37°C using a heating stirrer (GallenKamp Thermo, Cheshire, UK) and oxygenated with 95% O_2 and 5% CO_2 medical gas mixture (BOC Gases Medical, Surrey, UK), at a flow rate of 8 L/min, throughout the perfusion.

The digested liver tissue was maintained in iced-cold EMEM and scissor-minced following the removal of the cannulae and sutures. The released cell suspension was filtered through 2-ply cotton swabs (Shermond, Brighton, UK) and subsequently through a 200−µm nylon cell strainer (BD Biosciences, Durham, NC, USA). Hepatocytes were washed in ice-cold EMEM containing 10% FCS using a low-speed centrifuge (Heraeus Instruments, Newport Pagnell, UK) at 50×*g* and 4°C for 5 min, and the centrifugation was triplicated at 4°C. The total number and viability of fresh hepatocytes were determined using a haematocytometer and the trypan blue exclusion technique with a standard upright light microscope (Leitz, Wetzlar, Germany). Red blood cells in hepatocyte pellet were lysed, if required, using sterile water for injection (Fannin Ltd., Dublin, Ireland) for 1 min at room temperature followed by centrifugation. The batch of hepatocytes with a viability of over 60% on trypan blue exclusion was used for experiments.

2.4 Hepatocyte Mono-culture and Co-culture

Culture vessels were pre-coated using aseptic techniques with 0.1-mg/mL rat tail collagen type I (Sigma-Aldrich, St Louis, MO, USA) in 1% acetic acid (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 2 h, followed by PBS rinse at 4°C for 24 h.

Fresh hepatocytes were plated onto collagen-coated, flat-bottom microplates at a density of 50,000 viable cells per cm^2 (hepatocyte mono-culture). The culture media consisted of phenol red-free William's E medium (WEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FCS, 10-mM HEPES, 2-mM L-glutamine, 0.1-µM dexamethasone (Sigma-Aldrich, St Louis, MO, USA), 0.1-µM human recombinant insulin (Sigma-Aldrich, St Louis, MO, USA), and 100-U/mL penicillin plus 100-µg/mL streptomycin. The cell morphology of hepatocyte mono-culture was examined using a standard inverted light microscope equipped with a digital SLR camera.

P6–8 MSCs were resuspended in hepatocyte culture medium and incubated on collagen-coated microplates at the predefined densities for 24 h. Fresh hepatocytes were seeded on top of MSCs monolayer at a constant density of 50,000 viable cells per cm^2 (hepatocyte co-culture). Hepatocyte mono-culture was used as control (control group), and MSCs mono-culture was used as blank control. The culture medium was refreshed on days 1, 3, 5, and 7, respectively. The cell morphology was examined using a standard inverted light microscope equipped with a digital SLR camera. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes (Eppendorf, Hamburg, Germany) and cryopreserved at −80°C for further experiments. Cell cultures were rinsed with one wash of PBS at room temperature for further experiments.

2.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 96-well Falcon® microplates (BD Biosciences, Durham, NC, USA) as described above for measuring mitochondrial succinic dehydrogenase activity representing hepatocyte overall viability. The cell cultures were rinsed with PBS, and incubated with plain WEM containing 0.5-mg/mL 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) solution, 200 µL per well, at 37°C for 4 h. The MTT solution was removed and replaced by 100% DMSO, 200 µL per well, to dissolve formazan produced by viable cells, and the microplate was vigorously shaken using a microplate shaker (Heidolph Instruments, Essex, UK) at 1,500 rpm for 1-2 min. The optical density (OD) was measured at 550 nm using an MRX microplate reader equipped with Revelation version 4.06 incorporating Core DLL version 4.06 and Statistics DLL version 4.06 (Dynex Technologies, Guernsey, UK). The reading of background MSCs mono-culture (blank control) was subtracted from that of hepatocyte co-culture to obtain the colorimetric OD of hepatocytes *per se* in co-culture (Mohajerani *et al.*, Cell Medicine, Part B of Cell Transplantation, 2010). The experiments were performed in quadruplicate and repeated in triplicate independently.

2.6 Sulforhodamine B Colorimetric Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 96-well Falcon® microplates as described above for measuring sulphorhodamine B (SRB) binding to basic amino acid residues on cell membrane surfaces representing overall cell attachment. The cell cultures were rinsed with PBS, and fixed in 50% ice-cold trichloroacetic acid (Sigma-Aldrich, St Louis, MO, USA), 50 µL per well, layering on top of plain WEM, 200 µL per well, at 4°C for 1 h. The microplates were rinsed with tap water, and the fixed cells were stained with 0.4% SRB solution (Sigma-Aldrich, St Louis, MO, USA) in 1% acetic acid (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 10 min. The wells were rinsed with 1% acetic acid and air-dried at room temperature. The SRB dye was solubilised in 20-mM unbuffered 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris-base) buffer (Sigma-Aldrich, St Louis, MO, USA), 200 µL per well, and

shaken on a microplate shaker (Heidolph Instruments, Essex, UK) at 1,500 rpm for 15 min. The OD was measured at 564 nm using the microplate reader. The reading of background MSCs mono-culture (blank control) was subtracted from that of hepatocyte co-culture to obtain the colorimetric OD of hepatocytes *per se* in co-culture (Mohajerani *et al.*, Cell Medicine, Part B of Cell Transplantation, 2010). The experiments were performed in quadruplicate and repeated in triplicate independently.

2.7 Albumin Enzyme-linked Immunoabsorbent Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well Falcon® microplates (BD Biosciences, Durham, NC, USA) as described above, and cell culture supernatants were collected for measuring free albumin secretion representing protein synthesis of hepatocytes. A human albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) with the solid-phase sandwich enzyme immunoassay technique was used to quantitate free albumin level. Nunc-Immuno 96 MicroWell solid plates (NUNC A/S, Roskilde, Denmark) were pre-coated with 1:100 affinity purified human albumin coating antibody, 100 µL per well, at room temperature for 60 min. The antibody solution was removed, and the wells were rinsed with five washes of detergent buffer. The nonspecific antibody binding sites were blocked with the blocking solution, 100 µL per well, at room temperature for 30 min. The blocking solution was removed, and the wells were rinsed with five washes of detergent buffer. Human reference serum albumin, at an initial concentration of 10,000 ng/mL, was serially diluted with sample/conjugate diluents to give the standards 6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL, whilst the sample/conjugate diluent was used as the zero standard (blank) for the albumin standard curve. Culture supernatants were centrifuged (Eppendorf, Hamburg, Germany) at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 20 folds) and incubated in the assigned wells, 100 µL per well, at room temperature for 60 min. The wells were rinsed with five washes of detergent buffer. The bound albumin was detected by 1:150,000 horseradish peroxide (HRP) detection antibody, 100 µL per well, at room temperature for 60 min. The HRP detection antibody was removed, and the wells were washed with were rinsed with five washes of detergent buffer. HRP chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB), 100 µL per well, was added, and the plate was incubated in the dark and at room temperature for 15 min. The enzymatic colour reaction was stopped by adding 0.18-M sulphuric acid (Sigma-Aldrich, St Louis, MO, USA), 100 µL per well. The OD was measured at 450 nm using the microplate reader. The albumin concentration (ng/mL) in each sample was determined using the albumin standard curve, and albumin secretion of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.

2.8 Urea Colorimetric Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well microplates as described above, and cell culture supernatants were collected for measuring urea synthesis representing nitrogen detoxification of hepatocytes. QuantiChrom™ urea assay kit (BioAssay Systems, Hayward, CA, USA) was used to quantitate urea level. Culture supernatants were centrifuged at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 50 folds) and transferred into a clear flat-bottom 96-well plate, 5 μL per well. Freshly prepared urea assay working solution was added, 200 μL per well, and the plate was incubated at room temperature for 20 min. Human reference serum urea, at an initial concentration of 50 mg/dL, was serially diluted with distilled water to give the standards 3.125, 6.25, 12.5, 25, and 50 mg/dL, whilst distilled water was used as the zero standard (blank) for the urea standard curve. Distilled water (blank control), urea standard, and fresh hepatocyte culture media (control) were assayed using the same protocol. The OD was measured at 520 nm using the microplate reader. The corrected urea concentration (mg/dL) in each sample was calculated using the urea standard curve, and urea synthesis of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.

2.9 Caspase-cleaved CK18 Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well microplates as described above, and cell culture supernatants were collected for measuring apoptosis-associated K18Asp396 (M30) neo-epitope of soluble caspase-cleaved CK18 (CCK18) representing caspase-mediated apoptosis of hepatocytes. M30 CytoDeath™ ELISA kit (PEVIVA AB, Bromma, Sweden) was used to quantitate soluble CCK18 level (Figure 2.2, left panel). Human reference CCK18 solutions, prepared at 250, 1,000, 3,000 U/L, were used as standards, whilst the sample/conjugate diluent was used as the zero standard (blank) for the CCK18 standard curve. Culture supernatants were centrifuged at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 20 folds) and incubated in the assigned wells, 25 μL per well, with HRP conjugate, 75 μL per well, on a microplate shaker, 600 rpm, at room temperature for 4 h. The HRP detection antibody was removed, and the wells were rinsed with five washes of detergent buffer. Chromogenic substrate TMB, 200 μL per well, was added, and the plate was incubated in the dark and at room temperature for 20 min. The enzymatic colour reaction was stopped by adding 0.18-M sulphuric acid, 50 μL per well. The OD was measured at 450 nm using the microplate reader. The CCK18 concentration (U/L) in each sample was calculated using the CCK18 standard curve, and soluble CCK18 release of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.

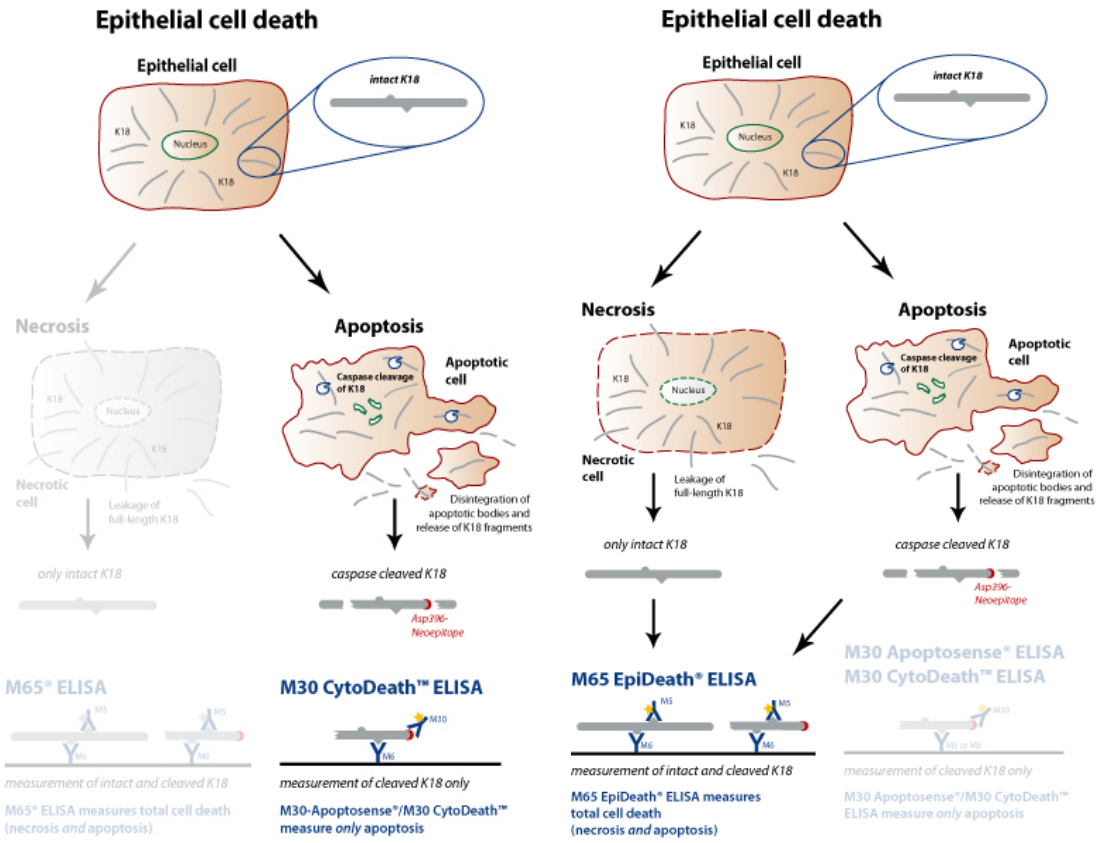

Figure 2.2 Caspase-mediated apoptosis and total cellular death (necrosis and apoptosis) of hepatocytes as represented by CCK18 (left panel) and CK18 (right panel). Adapted from *http://www.peviva.se*.

2.10 CK18 Assay

Hepatocyte mono-culture and co-culture were performed on collagen-coated, flat-bottom 24-well microplates as described above, and cell culture supernatants were collected for measuring soluble CK18 representing total cell death of hepatocytes. M65 EpiDeath® ELISA kit (PEVIVA AB, Bromma, Sweden) was used to quantitate soluble CK18 level (Figure 2.2, right panel). Human reference CK18 solutions, prepared at 200, 400, 800, 1,200, 2,000, 3,000, 5,000 U/L, were used as standards, whilst the sample/conjugate diluent was used as the zero standard (blank) for the CK18 standard curve. Culture supernatants were centrifuged at 1,500 rpm for 1 min to pellet any cell debris, and the samples were appropriately diluted (generally 50 folds) and incubated in the assigned wells, 25 µL per well, with HRP conjugate, 75 µL per well, on a microplate shaker, 600 rpm, at room temperature for 4 h. The HRP detection antibody was removed, and the wells were washed with were rinsed with five washes of detergent buffer. Chromogenic substrate TMB, 200 µL per well, was added, and the plate was incubated in the dark and at room temperature for 20 min. The enzymatic colour reaction was stopped by adding 0.18-M sulphuric acid, 50 µL per well. The OD was measured at 450 nm using the microplate reader. The CK18 concentration (U/L) in each sample was calculated using the CK18 standard curve, and soluble CK18 release of hepatocytes in mono- and co-culture was normalised to one million seeded viable hepatocytes. The experiments were performed in duplicate and repeated in triplicate independently.

Induction of apoptosis in cultured hepatocytes will result in massive release of CCK18 and secondary release of CK18 at a later time point, and consequently increase CCK18/CK18 ratio. In contrast, induction of necrosis will almost exclusively result in massive release of non-cleaved CK18, and consequently give rise to a low CCK18/CK18 ratio. The ratio of CCK18 to CK18 was, thus, calculated to determine the mode of death for hepatocytes *in vitro*; a ratio over 0.40 indicated that hepatocytes underwent apoptosis mainly, and *vice versa* (Kramer *et al.*, 2004).

2.11 Statistical Analysis

GraphPad Prism 6 programme (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. All continuous data were expressed as mean ± standard deviation (SD), and the means were compared using the one-way repeated measures analysis of variance (ANOVA), the Fisher's least significant difference (LSD) test, or two independent samples student-*t* test unless specified otherwise. A two-tailed *P*-value less than 0.05 was considered statistically significant.

CHAPTER 3 OPTIMISATION OF HUMAN HEPATOCYTE AND MESENCHYMAL STEM CELL *IN VITRO* CO-CULTURE SYSTEM

3.1 Introduction

3.1.1 *Limitations of hepatocyte mono-culture*

Once isolated and cultivated *in vitro*, primary hepatocytes in mono-culture lose their proliferative potential although the liver *in vivo* is well known for its potent regenerative capacity as seen in the Prometheus myth. Hepatocytes cultured *in vitro* switch from the proliferative response to the inflammatory response, as mediated by downregulated expression of NFκB (Chaisson *et al.*, 2002; Fredriksson *et al.*, 2011; Malato *et al.*, 2012) and upregulated expression of MAPK/ERK (Roberts *et al.*, 2000; Frémin *et al.*, 2007) signalling pathways. As a result, primary hepatocytes in standard monolayer culture show a rapid phenotypic de-differentiation and metabolic regression (Mizumoto *et al.*, 2008).

Hepatocytes also undergo epithelial-to-mesenchymal transition (EMT) in the absence of specific favourable growth factors, such as HGF (Xia *et al.*, 2006), whilst TGF-β, a potent profibrogenic factor, is known to be the most potent mediator of hepatocyte EMT as for other epithelial cell lines (Dooley *et al.*, 2008). Hepatocyte EMT is also believed to be one of the major aetiologies of liver fibrosis (Breitkopf *et al.*, 2006) aside from dysregulated proliferation of liver NPCs. *In vitro* cultured hepatocytes, if unattached, will undergo spontaneous apoptosis (Vanhaecke *et al.*, 2004). This spontaneous event is controversially thought to result from the accumulation of pre-existing nitric oxide (Canová *et al.*, 2008) and can also be induced by TNF or TGF-β (Roberts *et al.*, 2000).

3.1.2 *Heterotypical co-culture of hepatocytes with hepatic NPCs*

Hepatic NPCs, which consist primarily of Kupffer cells, sinusoidal endothelial cells, and stellate cells, play a regulatory role in hepatocyte maintenance, proliferation (Harada *et al.*, 2003), apoptosis (Chaisson *et al.*, 2002), maturation (Mitaka *et al.*, 1999; Melgert *et al.*, 2000), and regeneration (Sakuda *et al.*, 2002; Azuma *et al.*, 2003). The regulatory effects of NPCs on hepatocytes are believed to be mediated by paracrine factors, such as HGF (Michalopoulos *et al.*, 1999), EGF (Michalopoulos *et al.*, 1999), TGF-β (Date *et al.*, 1998), TNF (Shinozuka *et al.*, 1996), IGF, IL-1 (Boulton *et al.*, 1997), IL-6, BMP-6, and ECM (Vrochides *et al.*, 1996). However, co-culture with liver-derived NPCs stimulates hepatocyte DNA synthesis, depending on cell-to-cell interactions rather than growth factors. NPCs co-culture can augment liver-specific metabolic functions of hepatocytes exposed to ALF serum (Nedredal *et al.*, 2007). NPCs co-culture also helps two-dimensional sheets of hepatic progenitor cells to generate functionally differentiated three-dimensional liver tissue (Ogawa *et al.*, 2004). It is possible that hepatocytes and hepatic NPCs isolated from a single donor could be co-transplanted into a given recipient with more benefits.

3.1.3 *Heterotypic co-culture of hepatocytes with MSCs*

Co-culture of seed cells with MSCs appears to be a promising solution to modify cellular replacement therapy for metabolic disorders, such as diabetes mellitus and liver-based metabolic diseases, as heterotypic interaction with MSCs is known to be crucial for survival and functionality of epithelial cells *in vitro* (Gómez-Aristizábal *et al.*, 2009). Human MSCs enhance survival, metabolic function, and *in vivo* angiogenesis of isolated pancreatic islets by secreting trophic factors, such as VEGF (Park *et al.*, 2010), and by depositing ECM (Hematti *et al.*, 2013). Furthermore, Ito and his colleagues (2010) reported that co-transplantation with MSCs improved the morphology and function of islet grafts by promoting re-vascularisation *in vivo*. Among these exploratory studies, BM-MSCs are the most frequently used supportive cells as these cells are more easily expanded *in vitro* as documented in current literature (Karaoz *et al.*, 2011; Yeung *et al.*, 2012).

3.1.3.1 Heterotypic co-culture of hepatocytes with BM-MSCs

In the field of HCT, MSCs also show a favourable supportive effect on hepatocytes in long-term co-culture *in vitro* (Corlu *et al.*, 1997). BM-MSCs are still the most frequently used MSCs for hepatocyte co-culture. BM-MSCs maintain and improve hepatocyte morphology and metabolic functionality by the synergistic effects of soluble factors, cell-to-matrix, and cell-to-cell communications (Ijima *et al.*, 2008; Gu *et al.*, 2009[a]; Gu *et al.*, 2009[b]; Gu *et al.*, 2009[c]).

Co-transplantation of hepatocytes and BM-MSCs was also attempted in preclinical studies. When co-encapsulated with BM-MSCs, hepatocytes could be traced up to 4 months following transplantation in rats (Liu and Chang, 2002). Taking all these studies together, BM-MSCs can improve cellular morphology, survival, and metabolic functions, such as albumin secretion, urea synthesis, and CYP450 activity, both *in vitro* and *in vivo*. It was, therefore, proposed that co-transplantation of iPS derived cells and MSCs might be a novel alternative to OLT, which is limited by a shortage of donors and immune rejection, for treating end-stage liver disease (Liu *et al.*, 2009).

3.1.3.2 Heterotypic co-culture of hepatocytes with UC- and AT-MSCs

Two other common sources of MSCs, namely, UC- and AT-derived MSCs, have also been investigated in the setting of preclinical study. Chao *et al.* (2008) reported that human UC-MSCs improved islet cell secretion of insulin over 3 months possibly by releasing a series of trophic factors, including IL-6, tissue inhibitors of metalloproteinase (MMP)-1 and -2, monocyte chemoattractant protein 1, growth related oncogene, HGF, IGF binding proteins 4, and IL-8. Gómez-Aristizábal and Davies (2012) showed that human UC perivascular cells containing UC-MSCs enhanced liver-specific gene expressions, such as albumin, urea, and CYP450, mainly through MSC-to-hepatocyte contact and partially through paracrine factors.

AT-MSCs, also called adipose stem cells, are a newer member of the MSCs family that was introduced in the early 21st century and less studied for co-culture with hepatocytes. Cavallari *et al.* (2012) preconditioned human AT-MSCs with a mixture of hyaluronic, butyric, and retinoic acids and successfully optimised co-transplantation of rat islet cells with MSCs in a diabetic rat model, in

which multiple growth factor signalling pathways, such as VEGF, kinase insert domain receptor transcript, and HGF, were activated. The soluble factors mediating the immunomodulatory effect of AT-MSCs also include TGF-β, chemokine (C-C motif) ligand (CCL) 2, CCL5, tissue inhibitor of MMP-1/2, and cyclooxygenase-2 (Kang *et al.*, 2008).

3.1.3.3 Optimal source of MSCs for hepatocyte co-culture

BM-, UC-, and AT-MSCs have their own advantages and disadvantages in the scenario of clinical transplantation practice (Table 3.1). It remains unknown in the current literature which source of MSCs, namely, BM, UC, or AT, is the optimal candidate for the purpose of hepatocyte co-culture, although BM-MSCs are most frequently used in co-culture experiments.

Generally, these three types of MSCs have similar morphology and immunophenotype. BM and AT have a significantly higher success rate of MSCs isolation as compared to UC (100% *vs.* 100% *vs.* 63%); AT has the highest colony frequency, but UC has the lowest; and UC has the highest proliferative capacity, but BM has the lowest (Kern *et al.*, 2006). The low success rate of UC-MSCs isolation can be improved by selecting cord blood unit with a volume of more than 90 mL and a harvest time within 2 hours after the donor's birth (Zhang *et al.*, 2011). It is also confirmed that BM-MSCs are more likely to become senescent through passages *in vitro*, as compared to UC- and AT-MSCs (Vidal *et al.*, 2012).

All MSCs share some consistent and reproducible gene expression profiles involved in ECM formation, such as fibronectin, ECM2, glypican 4, DNA-binding protein inhibitor 1, neurofibromin 1b, and homeobox (HOX) A5 and B6, but also exhibit some differential gene expression profiles (Wagner *et al.*, 2005). Human adult BM-, UC-, and AT-MSCs also exhibit similar immunomodulatory effects *in vitro*. A study in a canine model reported that AT-MSCs had a significantly higher proliferative potential and BM-MSCs secreted the highest level of VEGF; AT- and UC-MSCs exhibited a greater *in vitro* osteogenic capacity as compared to BM-MSCs (Kang *et al.*, 2012). Another *in vitro* study comparing human BM-, UC-, and AT-MSCs, with respect to surface antigen expression, differentiation potential, proliferative capacity, clonality, tolerance for ageing, and paracrine activity, showed that UC-MSCs had the highest rate of cell proliferation and clonality and a significantly lower level of senescence marker expression; human UC-MSCs reduced expression of proinflammatory cytokines, such as IL-1α, -6, and -8, in co-cultured, LPS-challenged rat alveolar macrophages (Jin *et al.*, 2013).

Interestingly, these three types of MSCs have a similar potential of hepatogenic differentiation and proliferation, while placenta-derived MSCs have the reportedly greatest potential (Lee *et al.*, 2012). Moreover, it has been reported that human UC-MSCs are more effective for improving ureagenesis while BM-MSCs is more supportive for CYP450 activity in co-cultured hepatocytes (Gómez-Aristizábal *et al.*, 2012).

Table 3.1 Overview of BM-, UC, and AT-MSCs

BM	UC blood/matrix	AT
● Invasive harvest	● Non-invasive	● Non-invasive as side product of liposuction
● Propagatable but reduced in aged donors	● Highly propagatable	● Highly propagatable
● Less homogenous stem cell population	● Homogenous stem cell population	● Heterogeneous cell population
● Limited source	● Limited source	● Relatively unlimited source
● Readily available	● Not readily available	● Readily available
● Most extensively studied, showing high-efficiency differentiation	● Extensively studied, showing less efficient differentiation	● Less studied, and not well documented

3.1.4 *Compromised functionality of steatotic hepatocytes and cryopreserved human hepatocytes*

3.1.4.1 Steatotic hepatocytes

Liver steatosis, also called fatty liver disease (FLD), refers to a pathological accumulation of lipid, mainly triglyceride acid, in liver cells. FLD is further classified as alcoholic and non-alcoholic subtypes. Early-stage FLD is usually reversible in most cases and thought to result primarily from imbalanced fatty acid metabolism secondary to abusive alcohol consumption, obesity (Fabbrini *et al.*, 2010), metabolic disorder, such as insulin resistance, and use of hormonal or cytotoxic agents. FLD patients are usually asymptomatic and exhibit no symptom or sign of liver injury if the liver remains well compensated. However, severe FLD has accompanying hepatocyte necrosis and inflammatory response, namely, steatohepatitis. This pathological condition can trigger the activation of stellate cells and lead to liver fibrosis (Dixon *et al.*, 2001).

The prevalence of non-alcoholic FLD is very high and variable in the general population, ranging from 14% to 34% (Browning *et al.*, 2004; Bedogni *et al.*, 2005). This figure is much higher in the Western population even without a history of alcohol abuse (Angulo, 2002). FLD is the most common cause of liver function test abnormality in European and North American populations. As FLD remains silent until the time of liver imaging or biopsy, fatty donor livers with a poor graft survival and functionality are frequently (estimated to be 25%) encountered in OLT practice (Marsman *et al.*, 1996). Donor livers assigned to HCT use are often rejected for OLT mainly due to the presence of serious liver steatosis (Baccarani *et al.*, 2003). Alexandrova *et al.* (2005) reported severe liver steatosis rather than long cold ischaemia time or older donor age was the primary risk factor of a low hepatocyte isolation efficiency. Moderate to severe steatosis (>10% steatotic hepatocytes) was reported to be associated with a low hepatocyte isolation yield from liver resection specimens (Alexandre *et al.*, 2002) although mild steatosis (≤10% steatotic hepatocytes) did not result in a poor harvest of hepatocytes (Alexandre *et al.*, 2002; Richert *et al.*, 2004). Bonora-Centelles *et al.* (2010) reaffirmed that donor livers with a cold ischaemia time over 15 hours or with underlying serious steatosis should not be accepted for HCT as these donor livers offered poor-

quality hepatocytes in terms of cellular viability, attachment efficiency, and metabolic functionality. Severely steatotic hepatocytes cannot tolerate cryopreservation and subsequent thawing, and are consequently subject to a further cell loss and metabolic impairment (Terry *et al.*, 2005).

Sagias *et al.* (2010) added N-acetylcysteine (NAC), a widely used antioxidant for treating paracetamol-induced liver toxicity, into the perfusion solution of hepatocyte isolation and improved the viability of severely steatotic hepatocytes. The hepatoprotective effect of NAC is thought to result from the elimination of excessive production of ROS during ischaemia-reperfusion (Kohli *et al.*, 2007). Omega-3 polyunsaturated fatty acid was reported to reverse mitochondrial injury of steatotic hepatocytes by upregulating mitofusin 2 gene encoding mitochondrial membrane protein (Zhang *et al.*, 2011). Glucagon-like peptide-1 analogue can reduce hepatocyte steatosis and improve cell survival by enhancing unfolded protein response and promoting macroautophagy (Sharma *et al.*, 2011). However, it remains yet to be investigated how to improve the morphology and metabolic function of steatotic hepatocytes in the long term, especially after transplantation.

3.1.4.2 Cryopreserved hepatocytes

Hepatocytes can be cryopreserved and stored in a ‘‘cell bank’’ for several days, months, or even years following isolation, and cryopreserved hepatocytes can be subsequently thawed, allowing repeated or emergency transplantation on demand (Terry *et al.*, 2010). A major limitation of cryopreservation for standard hepatocyte monolayer culture is that post-cryopreserved hepatocytes exhibit a poor cell attachment and consequent metabolic impairment on thawing. Intracellular ice formation and exposure to hyperosmotic solutions are two major causes for hepatocyte injury following cryopreservation and subsequent thawing. These injuries can damage hepatocyte cytoplasmic membrane and result in massive loss of cytosolic proteins. Therefore, cryopreserved/thawed hepatocytes have a significant reduction in albumin secretion, urea synthesis, and CYP450 activity as compared to the pre-freezing baseline. Severity of hepatocyte cryopreservation/thawing injury depends on the condition of the donor liver tissue, such as age and well-being of the donor, concomitant liver condition (e.g., liver steatosis), and time lengths of cold and warm ischaemia (Terry *et al.*, 2005).

A large number of studies reported optimisation of hepatocyte cryopreservation protocols, aiming to improve post-freezing/thawing hepatocyte viability and metabolic function. These modified strategies include pre-culture of liver cell suspension, pre-incubation with antioxidants (Terry *et al.*, 2006), standardisation of hepatocyte concentration and cryovial use, and optimisation of cryoprotectants (e.g., long-chain oligosaccharide; Miyamoto *et al.*, 2006) and cooling/thawing procedures. As hepatocytes become apoptotic following detachment from ECM, cryopreservation of hepatocytes in artificial ECM, such as collagen and high molecular weight polymer, has also been investigated. Canaple *et al.* (2001) reported that cryopreservation of hepatocytes encapsulated in multicomponent capsules, composed of a polyelectrolyte complexation of sodium alginate, cellulose sulphate and poly(methylene-co-guanidine) hydrochloride, maintained liver-specific metabolic function of frozen hepatocytes for up to 4 months as compared to unfrozen cells. Interestingly, it has

been reported that co-encapsulation of hepatocytes with MSCs can improve cell graft survival and liver-specific metabolic function both *in vitr*o (Liu and Chang, 2003) and *in vivo* (Shi *et al.*, 2009). Therefore, it is possible that co-encapsulated MSCs should protect hepatocytes from freezing/ thawing injury. However, it remains unknown whether these protective effects can be maintained in the longer term, especially after thawing.

3.1.5 *Antiapoptotic effect of MSCs co-culture on hepatocytes in vitro*

MSCs co-culture has a significant modulatory effect on the cell cycle of hepatocytes. Gu *et al.* (2009[b]; 2009[c]) reported that a larger percentage of hepatocytes co-cultured with BM-MSCs were accumulated in the G_2/S phase, with a smaller percentage in the G_0/G_1 phase, as compared to mono-cultured hepatocytes. It suggests that MSCs co-culture facilitates the bypass of G_1/S checkpoint (Pok *et al.*, 2013). This checkpoint is known to be regulated by protein 53 (p53), a tumour suppressor. p53 can arrest cell growth by holding the cell cycle at the G_1/S checkpoint, which allows sufficient time for the cell to recognise and repair DNA damage; if the damage is irreparable, p53 will initiate cell apoptosis (Jensen *et al.*, 1998).

MSCs transplantation has been investigated in preclinical and clinical studies to ameliorate liver ischaemia/reperfusion injury (Pan *et al.*, 2012; Sun *et al.*, 2012) and chemically-induced liver fibrosis (Manuelpillai *et al.*, 2010). Potential contribution of MSCs may result from two aspects, including transdifferentiation into hepatocytes *in vivo* and promoting liver regeneration by paracrine mechanisms. Yan *et al.* (2009) reported that transplantation of human UC-MSCs could reduce serum aminotransferase level and hepatocyte denaturation by inhibiting hepatocyte apoptosis and promoting hepatocyte proliferation as evidenced by hepatocyte tracing and proliferating marker labelling. Antiapoptotic and antifibrotic effects of MSCs *in vivo* may derive from the contribution of HGF signalling, which modulates activation, apoptosis, and TGF-β signalling downregulation of hepatic stellate cells. Therefore, it is possible that MSCs co-culture exerts trophic and protective effects on hepatocytes by inhibiting hepatocyte spontaneous and chemically-induced apoptosis. This possibility is clinically significant for the use of HCT in the setting of ALF as transplanted hepatocytes will be exposed to a large number of circulating and regional (pro)inflammatory factors, a great majority of which are also proapoptotic, such as IL-6 and TNF-α, present in ALF patients.

3.1.6 *Chapter objectives*

- *Co-culture of hepatocytes and autologous liver NPCs*

To investigate whether co-culture with autologous liver NPCs can also be trophic for hepatocytes *in vitro*.

- *Optimisation of MSCs source for hepatocyte co-culture*

To compare hepatotrophic effect of BM-, UC, and AT-MSCs on co-cultured primary human

hepatocytes, at an optimal MSC:hepatocyte ratio, with respect to cellular viability, cell attachment, and liver-specific metabolism.

- *Co-culture steatotic/cryopreserved hepatocytes with MSCs*

To investigate whether MSCs can also exert trophic effects on co-cultured steatotic or cryopreserved hepatocytes.

- *Contribution of soluble factors to MSCs co-culture hepatotrophic effect*

To investigate whether soluble factors released from MSCs contribute to hepatotrophic effect of MSCs co-culture.

- *Antiapoptotic effect of MSCs co-culture*

To investigate whether MSCs can protect co-cultured hepatocytes from spontaneous and chemically-induced cell apoptosis.

3.2 Materials and Methods

3.2.1 Isolation of human liver-derived NPCs

Human liver-derived NPCs were isolated using a modified protocol described by Najimi *et al.* (2007). Briefly, the donor liver tissue was processed as described in ***Section 2.3*, *Primary Harvest of Human Hepatocytes***. Liver cell suspension was centrifuged at 50×g and 4°C for 5 min to pellet hepatocytes, and the supernatant was collected and further centrifuged for three cycles at 1,500 rpm and 4°C for 5 min. The total number and viability of liver cells in the supernatant were determined using trypan blue exclusion with a hematocytometer and a light microscope. Red blood cells in the cell pellet were lysed using sterile water for injection for 1 min at room temperature, followed by centrifugation. The cell suspension was resuspended in the MSCs expansion media and plated onto a non-tissue culture treated, 30-mm, polypropylene Petri dishes (Thermo Fisher Scientific, Ltd, Loughborough, UK) at a density of 60,000 cells per cm^2. As hepatocytes could adhere to polypropylene, floating hepatocytes (90%) were eliminated when the cell culture media were refreshed 4 h following plating. The cell culture was rinsed with sterile PBS and used for experiments.

3.2.2 Subculture of human MSCs and adult dermal fibroblasts

Human AT-, BM-, and UC-MSCs were subcultured as described in ***Section 2.2*, *Subculture of MSCs***. Human adult-derived dermal fibroblasts (ADFs), a common mesenchyme-derived cell line, were originally extracted by Life Technologies Corporation, Carlsbad, CA, United States from a single human donor's foreskin through mechanical and enzymatic digestion. These cells were used as a random control for MSCs. Primary cultures was expanded for one passage before cryopreservation. Cryopreserved human ADFs (approximately 0.5 million cells per mL per vial) were quickly swirled and thawed in a 37°C water bath for 1 min. The cell suspension was immediately transferred into a 50-mL sterile polypropylene conical tube containing 20-mL pre-warmed culture media and centrifuged at 1,500 rpm for 5 min. The cell pellet was subsequently resuspended in pre-warmed culture media and plated onto a 25-cm^2 tissue culture flask (NUNC A/S, Roskilde, Denmark) at a density of 5,000 cells per cm^2 in a humidified incubator, in an atmosphere of 95% O_2 and 5% CO_2 and at 37°C. The ADFs expansion culture media consisted of phenol red-free, low-glucose DMEM, 10% FCS, 2-mM L-glutamine, and 100-U/mL penicillin plus 100-μg/mL streptomycin, at a volume of 5 mL per T25 flask. The culture medium was replaced with fresh medium every 3–4 d.

On day 7 of culture (approximately 80% confluency), ADFs cultures were rinsed with PBS and detached by adding 1-mL 0.25% trypsin at 37°C for 5–10 min, and the cell dissociation was stopped by 10% FCS/DMEM. The cell suspension was replated and subcultured at a ratio of 1:3 using the same cell culture protocol. The cells were cryopreserved using a freezing container at −80°C, and the stocks were stored at −140°C in the culture media supplemented with 10% DMSO. The 4^{th}–6^{th}

passages (P4–6) of ADFs were used for further experiments. The total number and viability of ADFs for each passage were determined using the trypan blue exclusion technique with an inverted light microscope equipped with a digital SLR camera.

3.2.3 Isolation of human non-steatotic and steatotic hepatocytes

Liver steatosis was graded by an independent consultant liver histopathologist at Institute of Liver Studies, King's College Hospital NHS Foundation Trust, London, UK, using the standard four-grade semiquantitative evaluation scale (Franzén *et al.*, 2005): grade 0, no fat deposition in hepatocytes; grade 1 (mild), fat deposition in less than 33% of hepatocytes; grade 2 (moderate), fat deposition in 33%–66% of hepatocytes; and grade 3 (severe), fat deposition in more than 66% of hepatocytes. Non-steatotic donor liver tissues were processed as described in ***Section 2.3*, *Primary Harvest of Human Hepatocytes***, while moderately steatotic donor liver tissues were processed similarly, except for the addition of 5-mM NAC (PLIVA Pharma, Ltd., Hampshire, UK), a potent scavenger of ROS precursors into the first perfusion buffer (Ca^{2+}-free HBSS) of the standard collagenase digestion technique as previously reported by Sagias *et al.* (2010). The total number and viability of fresh hepatocytes were determined using a hematocytometer and the trypan blue exclusion technique with a light microscope. Red blood cells in hepatocyte pellet were lysed using sterile water for injection for 1 min at room temperature, followed by centrifugation. Batches of hepatocytes with a viability of over 60% on trypan blue exclusion were used for experiments.

3.2.4 Cryopreservation and thawing of human hepatocytes

Freshly isolated hepatocytes were cryopreserved using a standard controlled-rate freezing protocol as previously reported by Mitry *et al.* (2010). Hepatocytes were resuspended in cryopreservation solution at a final density of 1.0×10^7 cells per mL. The cryopreservation solution, consisting of 1 part of 50% glucose (Hameln Pharmaceuticals Ltd., Gloucester, UK), 1 part of clinical grade 100% DMSO, and 8 parts of ViaSpan™ University of Wisconsin (UW) solution (Bristol-Myers Squibb Pharmaceutical Limited, Dublin, Ireland), was chilled on an ice pack. Hepatocyte suspension (5 mL) was transferred into a sterile 10-mL cryovial (Thermo Fisher Scientific) using a 10-mL syringe (BD Biosciences) chilled on an ice pack and immediately placed flat into the controlled-rate freezer connected to a liquid nitrogen container (Kryo 10, series III; Planer Products, Ltd., Middlesex, UK) at a pressure stabilised between 34.5 and 48.3 kPa. The cryovial holder and frame were pre-chilled at 4–8°C for 30–60 min prior to use. A modified stepwise controlled-rate freezer programme was started to produce a linear temperature decrease and prevent cell damage from latent heat of fusion during water crystallisation (Diener *et al.*, 1993). The start temperature was 8°C, and the end temperature was −140°C over 60-minute freezing. Frozen cryovials were immediately transferred on dry ice to the −140°C cell storage tank.

Cryopreserved hepatocytes were defrosted using a modified protocol as previously reported by Steinberg *et al.* (1999). Frozen hepatocyte suspension (5 mL per vial) was briefly and gently thawed

in a 37°C water bath, and immediately transferred into a 250-mL Falcon® polypropylene conical tube (BD Biosciences) containing 50-mL ice-cold thawing solution at a slow rate using the aseptic technique. The thawing solution consisted of 1 part of 20% human serum albumin (Baxter Healthcare Ltd., Compton, UK) and 9 parts of EMEM. Hepatocyte suspension was pelleted at 50×g and at 4°C for 5 min and re-washed in 20-mL ice-cold 2% HSA/EMEM solution. The total number and viability of fresh hepatocytes were determined using a hematocytometer and the trypan blue exclusion technique with a standard upright light microscope. Batches of hepatocytes with a viability of over 60% on trypan blue exclusion was used for experiments.

3.2.5 Hepatocyte co-culture protocols

3.2.5.1 Hepatocyte co-culture with liver NPCs

Floating hepatocytes were eliminated from the supernatant cell culture by refreshing the cell culture media 4 h after plating. Hepatocyte mono-culture and co-culture with liver NPCs at a ratio of 10:1 were established as described in ***Section 2.4*, *Hepatocyte Mono-culture and Co-culture***. The seeding density of hepatocytes was fixed at 50,000 viable cells per cm^2. Hepatocyte mono-culture was used as control, and NPCs mono-culture was used as a blank control.

3.2.5.2 Optimisation of MSCs source

Fresh non-steatotic hepatocytes were co-cultured with P6–8 human AT-, BM-, and UC-MSCs, as well as P4–6 human ADFs, as described in ***Section 2.4*, *Hepatocyte Mono-culture and Co-culture***, to determine the optimal MSCs source for hepatocyte co-culture. The seeding density of MSCs and ADFs was 20,000 viable cells per cm^2, and that of hepatocytes was 50,000 viable cells per cm^2, at a MSC/ADF:hepatocyte ratio of 1:2.5. Hepatocyte mono-culture was used as control, hepatocyte co-culture with human ADFs was used as random control, and MSCs mono-culture was used as a blank control.

3.2.5.3 Optimisation of MSC:hepatocyte ratio

P6–8 optimal source-derived MSCs were co-cultured with fresh non-steatotic hepatocytes as described in ***Section 2.4*, *Hepatocyte Mono-culture and Co-culture***, to determine the optimal MSCs:hepatocytes ratio for hepatocyte co-culture. The seeding density of hepatocytes was fixed at 50,000 viable cells per cm^2, and those of the optimal source-derived MSCs were predetermined to be 50,000, 20,000, 10,000, and 5,000 viable cells per cm^2, at a ratio of 1:1, 1:2.5, 1:5, and 1:10, respectively. Hepatocyte mono-culture was used as control, and MSCs mono-culture was used as blank control.

3.2.5.4 Co-culture of cryopreserved/steatotic hepatocytes with MSCs

P6–8 optimal source-derived MSCs were co-cultured with cryopreserved non-steatotic, as well as fresh non-, mildly, moderately, and severely steatotic hepatocytes as described in ***Section 2.4*, *Hepatocyte Mono-culture and Co-culture***. The seeding density of hepatocytes was fixed 150,000 viable cells per cm^2, with that of the optimal source-derived MSCs at the optimal MSC:hepatocyte ratio. Hepatocyte mono-culture was used as control, and MSCs mono-culture was used as blank control.

3.2.5.5 Indirect co-culture of hepatocytes with MSCs

Indirect co-culture using Transwell plates Indirect co-culture of hepatocytes with the optimal source-derived MSCs using Transwell® Permeable Supports (Corning Incorporated, Corning, NY, USA) was performed to investigate the contribution of MSCs-derived soluble factors to the MSCs co-culture hepatotrophic effect. Hepatocytes were separated from MSCs in the Transwell® co-culture system to exclude possible effects of ECM and direct MSC-to-hepatocyte contact on contribution of soluble factors. The optimal source-derived MSCs were plated onto Transwell® permeable culture inserts at a density equalling to that at the optimal MSC:hepatocyte ratio, and pre-cultured with hepatocyte culture media for 24 hours. Fresh hepatocytes were mono-cultured as described in ***Section 2.4*, *Hepatocyte Mono-culture and Co-culture***, and MSCs-plated culture inserts were transferred into hepatocyte mono-culture using aseptic techniques with hepatocyte mono-culture alone as control.

MSCs co-culture CM Soluble trophic factors released from MSCs in co-culture are likely to be regulated by ECM and direct MSC-to-hepatocyte contact. Indirect co-culture of hepatocytes with the optimal source-derived MSCs co-culture CM was performed to investigate whether hepatotrophic effect of MSCs co-culture was mediated by paracrine mechanisms. The optimal source-derived MSCs were co-cultured with fresh hepatocytes for 24 h at the optimal MSC:hepatocyte ratio as described in ***Section 2.4*, *Hepatocyte Mono-culture and Co-culture***. Fresh MSCs co-culture CM was collected to feed mono-cultured hepatocytes, with hepatocytes mono-cultured with fresh hepatocyte culture media as control. The reading of background MSCs co-culture CM (blank control) was subtracted from that of hepatocytes cultured with CM to obtain the actual reading of hepatocytes.

The culture media were refreshed on days 1, 3, 5, and 7, respectively. The cell morphology was examined using a standard inverted light microscope equipped with a digital SLR camera; hepatocyte co-culture with AT-MSCs was also examined using a laser capture microdissection system (Leica Microsystems, Wetzlar, Germany). Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and frozen at −80°C for further experiments. Cell cultures were rinsed with one-wash PBS at room temperature for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.

3.2.6 Staurosporine cytotoxicity assay

Staurosporine cytotoxicity assay was performed to investigate whether MSCs co-culture specifically inhibited hepatocyte apoptosis by targeting at caspase signalling pathway. Staurosporine is a prototypical ATP-competitive protein kinase inhibitor that is mainly used to induce cell apoptosis in biological experiments and reported to activate caspase-3 signalling (Barrachina *et al.*, 2002). Fresh non-steatotic hepatocyte mono-cultures were treated with 0- (blank control), 0.5-, 1-, 2.5-, 5-, and 10-μM staurosporine (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cell culture supernatants were collected for CCK18 and CK18 assays as described in ***Section 2.9*, *Caspase-cleaved CK18 Assay*** and ***Section 2.10*, *CK18 Assay***. Fresh hepatocytes were co-cultured with the optimal source-derived MSCs at the optimal ratio in the presence of staurosporine at the least concentration that induced significant increases in hepatocyte apoptosis and total death, as controlled by hepatocyte mono-culture in the absence of staurosporine. All experiments were performed in duplicate and repeated in triplicate independently.

3.2.7 General cellular activity and liver-specific metabolic function assays

MTT assay was performed as described in ***Section 2.5*, *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay*** to determine hepatocyte mitochondrial dehydrogenase activity. SRB assay was performed as described in ***Section 2.6*, *Sulforhodamine B Colorimetric Assay*** to determine overall hepatocyte attachment. Albumin ELISA was performed as described in ***Section 2.7*, *Albumin Enzyme-linked Immunoabsorbent Assay*** to determine protein synthesis of hepatocytes. Urea colorimetric assay was performed as described in ***Section 2.8*, *Urea Colorimetry*** to determine nitrogen detoxification of hepatocytes. All experiments were performed in duplicate and repeated in triplicate independently.

3.2.8 Hepatocyte apoptosis and total death assays

CCK18 assay was performed as described in ***Section 2.9*, *Caspase-cleaved CK18 Assay*** to determine caspase-mediated hepatocyte apoptosis. CK18 assay was performed as described in ***Section 2.10*, *CK18 Assay*** to determine total hepatocyte death. The ratio of CCK18 to CK18 was also produced to determine the death mode of hepatocytes *in vitro*; a ratio of over 0.40 indicated that hepatocytes underwent apoptosis mainly, and necrosis *vice versa*. All experiments were performed in duplicate and repeated in triplicate independently.

3.3 Results

3.3.1 *Morphology of mono-cultured human liver NPCs, MSCs, and ADFs*

3.3.1.1 Human liver NPCs

Human liver NPCs showed a viability of 60–80% on trypan blue exclusion at the time of primary harvest. This mixed cell population could adhere to and grow on non-tissue culture treated plate surfaces. These cells were variable in cell size (10–40 μm), but uniformly spindle-shaped in contrast to polygonal- or round-shaped hepatocytes (Figure 3.1A). From day 7 onwards, these cells detached from the plate and could not be passaged.

3.3.1.2 Human AT-, BM-, and UC-MSCs

All three sources of MSCs showed a similar spindle-shaped, fibroblast-like morphology (20–40 μm) with double or multiple small projections after being seeded on non-tissue culture treated plate surface and reached 90% of confluency within 7 days of culture (Figure 3.1B–D). On confluency MSCs were polarised, with a small cell body and two slim projections in a homogenous manner. MSCs remained proliferative and attached from passages 6 to 8. Cell yield was approximately 1–2×10^6 cells per T75 culture flask, equal to a multiplication rate of 200–400%. The viability on trypan blue exclusion was over 99.5% for each passage and remained above 99% after freezing and thawing. UC-MSCs appeared to have a relatively rapid proliferation, while AT-MSCs exhibited a relatively large cell body on low confluency.

3.3.1.3 Human ADFs

Human ADFs also showed a spindle-shaped morphology (10–20 μm) with two slim projections after being seeded on non-tissue culture treated plate surface and reached 90% of confluency within 7 days of culture. On confluency ADFs were polarised, with a small cell body and two relatively shorter projections, as compared to MSCs, in a homogenous manner (Figure 3.1E). ADFs remained proliferative and attached from passages 4 to 6. Cell yield was approximately 0.5×10^6 cells per T25 culture flask, equal to a multiplication rate of 400%. The viability on trypan blue exclusion was over 90% for each passage and remained above 90% after freezing and thawing.

3.3.2 *Morphology of hepatocytes in mono-, direct co-, and indirect co-cultures*

3.3.2.1 Hepatocytes in mono-culture

Primary human hepatocytes attached to collagen-coated culture vessel surfaces within 4–6 hours, while a large number of hepatocytes remained unattached at the time of culture media replacement. Adherent hepatocytes at a low seeding density exhibited a round-shaped morphology at a size of below 10 μm. Primary hepatocytes aggregated into a few small-sized colonies containing 3–5 cells (Figure 3.2A); however, these colonies did not proliferate and could not be passaged. The

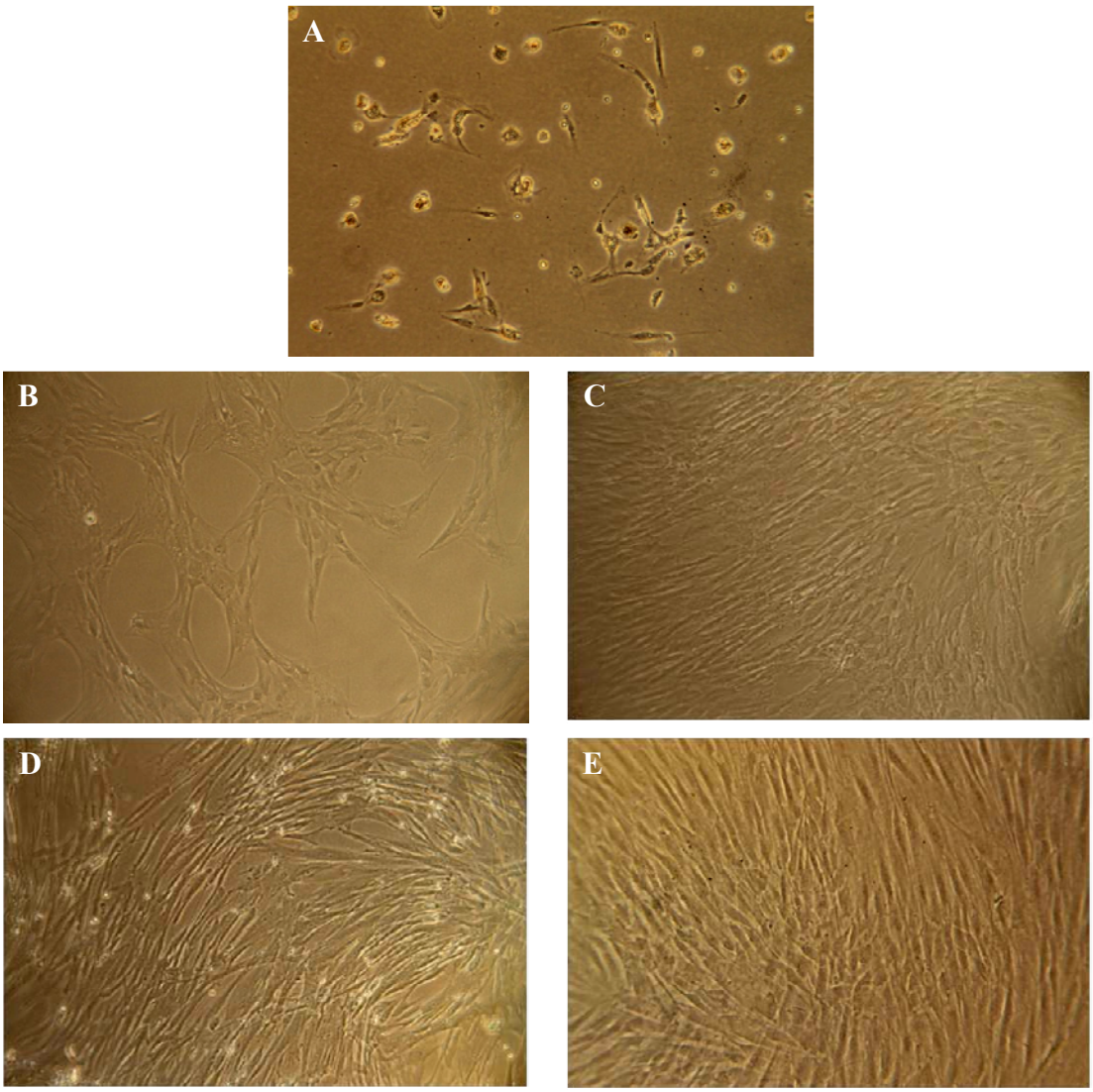

Figure 3.1 Morphology of human liver-derived NPCs (A), AT-MSCs (B), BM-MSCs (C), UC-MSCs (D), and ADFs (E) on inverted light microscopy (200×): (A) liver-derived NPCs were variable in cell size (10–40 μm) but uniformly spindle-shaped; (B–D) AT-, BM-, and UC-MSCs were homogenous and polarised, with a small cell body and two slim projections; and (E) ADFs were homogenous and polarised, with a small cell body and two relatively shorter projections.

viability of primary hepatocytes on trypan blue exclusion continued declining and neared zero on day 7 of *in vitro* culture.

3.3.2.2 Hepatocytes in co-culture with liver NPCs

Freshly isolated human hepatocytes attached to the liver NPCs monolayer within 4–6 hours, and a relatively small number of hepatocytes were detached from culture vessel surface at the time of culture media replacement. Adherent hepatocytes exhibited a morphology similar to those in mono-culture, while a greater number of hepatocytes aggregated and attached closely to liver NPCs over 7 days of co-culture (Figure 3.2B). The viability of primary hepatocytes co-cultured with autologous liver NPCs continued to decline but remained approximately 25% on day 7.

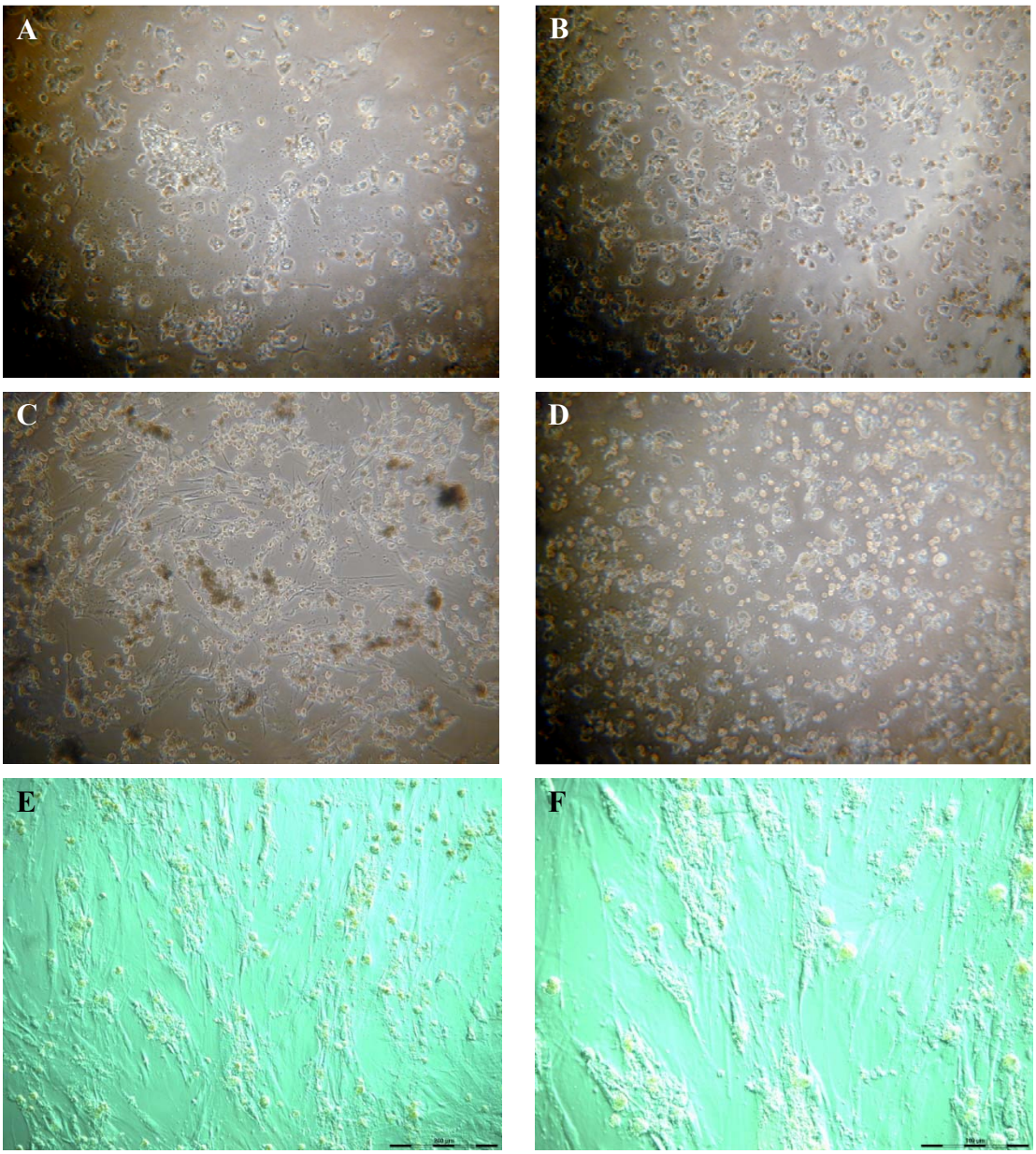

Figure 3.2 Morphology of human primary hepatocyte mono-culture (A), hepatocyte co-culture with liver-derived NPCs (B), hepatocyte co-culture with AT-MSCs (C), and hepatocytes indirectly co-cultured with AT-MSCs using Transwell culture system (D) on inverted light microscopy (200×) and hepatocyte co-culture with AT-MSCs on a laser capture microdissection system (E, 200×; F, 400×): (A) primary hepatocytes occasionally aggregated into a few small-sized colonies containing 3–5 cells; (B) a greater number of hepatocytes aggregated and attached closely to liver NPCs; (C) far more hepatocytes aggregated massively into larger, oval-shaped colonies and attached closely to MSCs; (D) a relatively greater number of attached hepatocytes aggregated into scattered, small-sized colonies; and (E,F) laser capture microscopy showed three-dimensional aggregation of hepatocytes (arrows) on top of MSCs monolayer.

3.3.2.3 Hepatocyte in direct co-culture with MSCs

The great majority of freshly isolated human hepatocytes attached to the MSCs monolayer within 2–4 hours, and only a few hepatocytes were detached from the culture vessel surface at the time of culture media replacement. Adherent hepatocytes exhibited a morphology similar to those in co-culture with liver NPCs, but far more hepatocytes aggregated into larger, oval-shaped colonies and attached close to MSCs over 7 days of co-culture (Figure 3.2C). Laser capture microscopy also

showed that aggregated hepatocytes were attached on top of MSCs monolayer in a three-dimensional manner (Figure 3.2E and F).

3.3.2.4 Hepatocytes in indirect co-culture with MSCs

Primary hepatocytes cultured with MSCs co-culture CM or indirectly co-cultured with MSCs on Transwell plates exhibited a morphology generally similar to those in mono-culture. Hepatocytes became adherent to collagen-coated culture vessel surfaces within 4–6 hours, and a large number of hepatocytes were detached at the time of culture media replacement. Adherent hepatocytes exhibited a morphology similar to those in mono-culture, and a relatively greater number of attached hepatocytes appeared to be aggregated into scattered, small-sized colonies (Figure 3.2D). The viability of primary hepatocytes on trypan blue exclusion also continued declining and neared zero on day 7 of *in vitro* culture.

3.3.3 *Hepatotrophic effect of NPCs co-culture*

3.3.3.1 Viability

Liver NPCs co-culture improved hepatocyte viability as compared to hepatocyte mono-culture throughout 7 days of culture (Figure 3.3A). On day 1, hepatocyte viability had significantly declined in both co- and mono-cultures as compared to the baseline (approximately 62%); however, the viability of hepatocytes co-cultured with liver NPCs was significantly higher than that of mono-cultured hepatocytes (co-culture *vs.* mono-culture: day 1, 40.6% ± 6.4% *vs.* 27.8% ± 3.1%; P <0.01). The viability of hepatocytes co-cultured with liver NPCs decreased at a relatively slower rate as compared to that of mono-cultured hepatocytes until day 7 (24.3% ± 4.2% *vs.* 1.5% ± 0.2%; P <0.01).

3.3.3.2 Mitochondrial dehydrogenase activity

Liver NPCs co-culture exhibited a significant hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to hepatocyte mono-culture throughout 7 days of culture (Figure 3.3B). Hepatocytes co-cultured with liver NPCs had a significantly higher MTT activity than control mono-culture from day 1 (2.35 ± 0.20 *vs.* 1.40 ± 0.10 OD units; P <0.01) until day 7 (1.53 ± 0.12 *vs.* 0.59 ± 0.10 OD units; P <0.01).

3.3.3.3 Cell attachment

Liver NPCs co-culture exhibited a significant hepatotrophic effect, with respect to hepatocyte attachment, as compared to control (Figure 3.3C). Hepatocytes co-cultured with liver NPCs had a significantly higher SRB cell attachment than control mono-culture from day 1 (2.83 ± 0.30 *vs.* 2.40 ± 0.22 OD units; P <0.05) until day 5 (2.39 ± 0.36 *vs.* 1.99 ± 0.25 OD units; P <0.05), with a similar cell attachment on day 7.

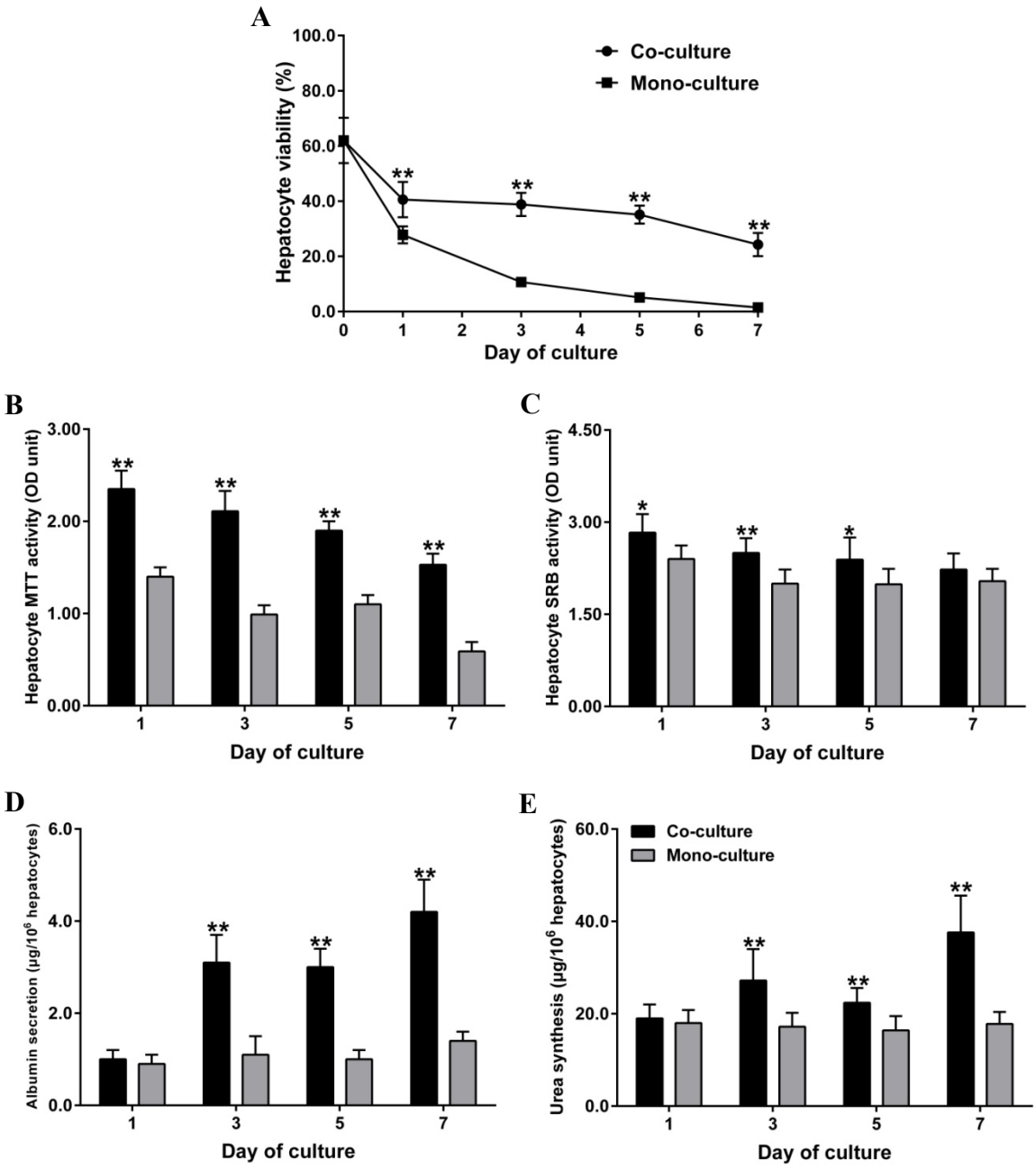

Figure 3.3 Viability (A), MTT activity (B), SRB cell attachment (C), albumin secretion (D), and urea synthesis (E) of hepatocytes co-cultured with liver NPCs *versus* hepatocyte mono-culture. Co-culture with liver NPCs showed significant hepatotrophic effect within 7 days of culture. All data were expressed as mean ± SD; **P* <0.05 and ***P* <0.01 (*n* = 6).

3.3.3.4 Albumin secretion

No albumin secretion was detected in liver NPCs mono-culture. Liver NPCs co-culture exhibited a significant hepatotrophic effect, with respect to albumin secretion, as compared to control (Figure 3.3D). Albumin secretion remained significantly higher in liver NPCs co-culture than that in control mono-culture from day 3 (3.1 ± 0.6 *vs.* 1.1 ± 0.4 μg/10^6 hepatocytes; *P* <0.01) until day 7 (4.2 ± 0.7 *vs.* 1.4 ± 0.2 μg/10^6 hepatocytes; *P* <0.01).

3.3.3.5 Urea synthesis

Liver NPCs co-culture also exhibited a significant hepatotrophic effect, with respect to urea synthesis, as compared to control (Figure 3.3E). Urea synthesis remained constantly higher in liver NPCs co-culture than that in control mono-culture from day 3 (27.2 ± 6.8 *vs.* 17.2 ± 3.0 μg/10^6 hepatocytes; P <0.01) until day 7 (37.6 ± 8.0 *vs.* 17.8 ± 2.6 μg/10^6 hepatocytes; P <0.05).

3.3.4 *Optimisation of MSCs/hepatocyte co-culture*

3.3.4.1 Optimal source of MSCs for hepatocyte co-culture

Proliferation of MSCs Cellular proliferation was similar among AT-, BM-, and UC-MSCs over 7 days of culture (Table 3.2). Mitochondrial dehydrogenase activity remained similar among three sources of MSCs from day 1 until day 7. SRB cell attachment also remained similar among these three sources of MSCs from day 1 until day 7.

Table 3.2 MTT and SRB activities (mean ± SD) of MSCs over 7 days of culture

	MTT (OD unit)				SRB (OD unit)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
AT-	1.26 ± 0.06	1.55 ± 0.12	1.92 ± 0.20	2.30 ± 0.24	1.22 ± 0.12	1.60 ± 0.23	2.14 ± 0.26	2.43 ± 0.23
BM-	1.12 ± 0.05	1.52 ± 0.10	2.02 ± 0.21	2.23 ± 0.21	1.28 ± 0.15	1.79 ± 0.19	2.20 ± 0.23	2.60 ± 0.34
UC-	1.14 ± 0.04	1.56 ± 0.12	1.68 ± 0.22	2.22 ± 0.19	1.18 ± 0.13	2.06 ± 0.19	2.16 ± 0.25	2.40 ± 0.27

Mitochondrial dehydrogenase activity ADFs co-culture showed a significant hepatotrophic effect as compared to control mono-culture; MSCs co-culture also exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to mitochondrial dehydrogenase activity, as compared to ADFs co-culture and control mono-culture (Figure 3.4A). Hepatocytes co-cultured with MSCs had a significantly higher MTT activity than those co-cultured with ADFs from day 3 (AT *vs.* BM *vs.* UC *vs.* ADFs *vs.* control, 1.62 ± 0.12 *vs.* 1.65 ± 0.13 *vs.* 1.69 ± 0.09 *vs.* 1.44 ± 0.07 *vs.* 1.23 ± 0.12 OD units; P <0.05) and control mono-culture from day 1 (1.65 ± 0.15 *vs.* 1.67 ± 0.16 *vs.* 1.58 ± 0.14 *vs.* 1.61 ± 0.17 *vs.* 1.39 ± 0.04 OD units; P <0.05) until day 7 (2.59 ± 0.25 *vs.* 2.49 ± 0.24 *vs.* 2.38 ± 0.20 *vs.* 1.69 ± 0.16 *vs.* 1.25 ± 0.15 OD units; P <0.01), respectively.

Cell attachment ADFs co-culture showed a limited hepatotrophic effect as compared to control mono-culture; MSCs co-culture exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to cell attachment as compared to ADFs co-culture and control mono-culture (Figure 3.4B). Hepatocytes co-cultured with MSCs had significantly higher SRB activity than those co-cultured with ADFs and control mono-culture from day 1 (3.07 ± 0.15 *vs.* 3.29 ± 0.16 *vs.* 3.24 ± 0.14 *vs.* 2.91 ± 0.17 *vs.* 2.75 ± 0.14 OD units; P <0.05) until day (3.58 ± 0.42 *vs.* 3.70 ± 0.30 *vs.* 3.63 ± 0.25 *vs.* 3.17 ± 0.26 *vs.* 3.00 ± 0.26 OD units; P <0.01).

Albumin secretion No albumin secretion was detected in ADFs and MSCs mono-culture. ADFs co-culture showed a significant hepatotrophic effect as compared to control mono-culture;

MSCs co-cultures also exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to albumin secretion, as compared to ADFs co-culture control mono-culture (Figure 3.4C). Hepatocytes co-cultured with MSCs had significantly higher albumin secretion than those co-cultured with ADFs and control mono-culture from day 3 (2.4 ± 0.3 *vs.* 2.5 ± 0.3 *vs.* 2.5 ± 0.3 *vs.* 2.0 ± 0.2 *vs.* 0.9 ± 0.2 μg/10^6 hepatocytes; P <0.05) until day 7 (4.6 ± 0.4 *vs.* 4.6 ± 0.3 *vs.* 4.3 ± 0.3 *vs.* 3.6 ± 0.3 *vs.* 1.4 ± 0.2 μg/10^6 hepatocytes; P <0.01).

Urea synthesis ADFs co-culture showed a significant hepatotrophic effect as compared to control mono-culture; MSCs co-cultures exhibited significant hepatotrophic effects, similar among three MSCs co-cultures, with respect to urea synthesis, as compared to ADFs co-culture and control mono-culture (Figure 3.4D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than those co-cultured with ADFs and control mono-culture from day 3 (23.4 ± 2.2 *vs.* 23.7 ± 1.9 *vs.* 23.0 ± 1.9 *vs.* 19.4 ± 2.4 *vs.* 15.8 ± 2.0 μg/10^6 hepatocytes; P <0.05) until day 7 (35.9 ± 3.0 *vs.* 36.4 ± 2.6 *vs.* 35.3 ± 2.5 *vs.* 32.1 ± 2.3 *vs.* 17.0 ± 2.4 μg/10^6 hepatocytes; P <0.01).

Overall AT-MSCs co-culture exhibited a significant hepatotrophic effect, similar to BM- and UC-MSCs co-cultures, as compared to ADFs co-culture and hepatocyte mono-culture. Due to the practical advantages of AT-MSCs in contrast to BM- and UC-MSCs, AT-MSCs were determined to be the optimal MSCs for further hepatocyte co-culture experiments.

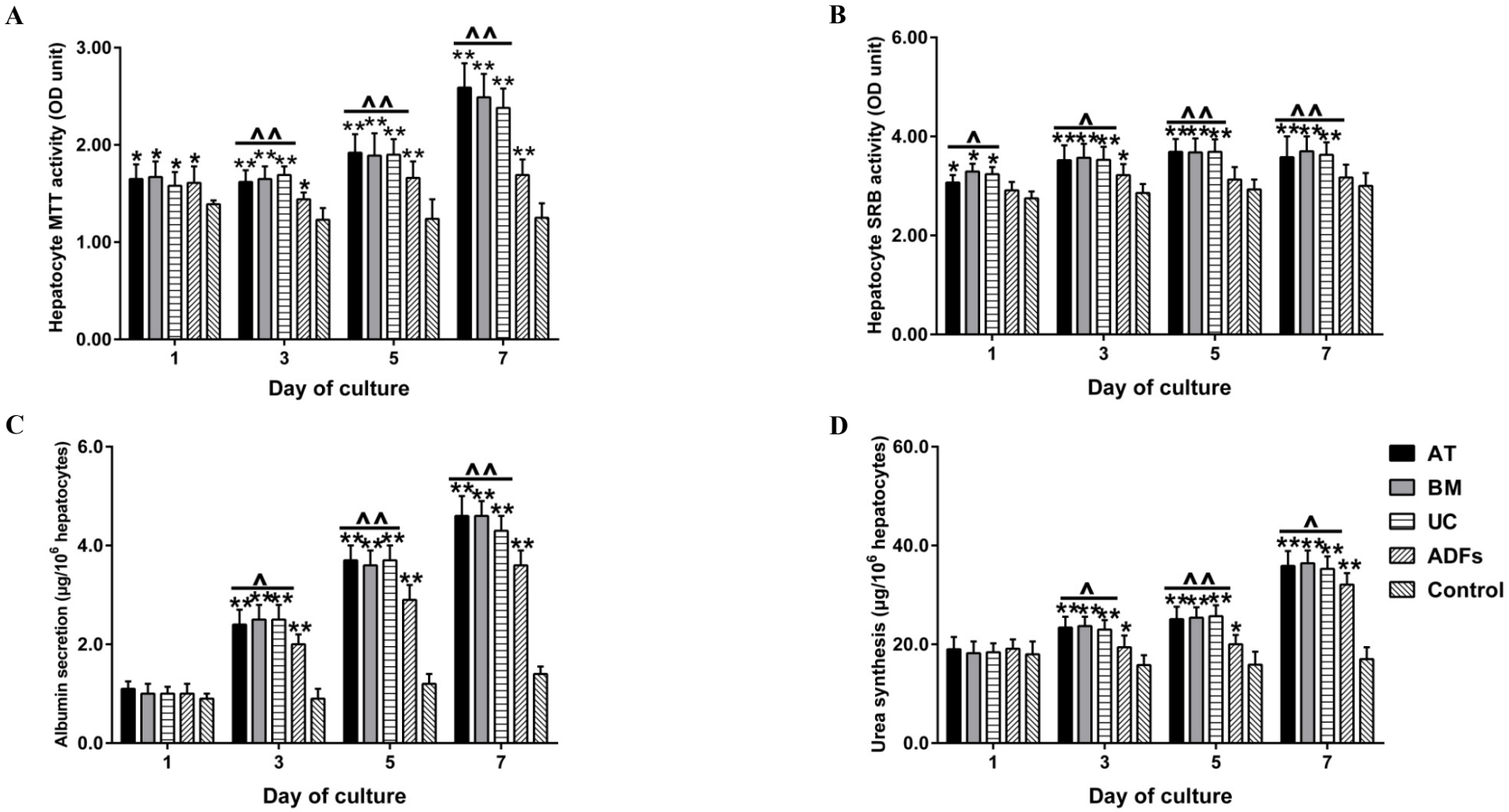

Figure 3.4 MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of hepatocytes co-cultured with AT-, BM-, and UC-MSCs *versus* hepatocytes co-cultured with ADFs and hepatocyte mono-culture. Co-culture with MSCs showed a significant hepatotrophic effect, similar among the three co-cultures, within 7 days of culture. All data were expressed as mean ± SD; **P* <0.05 and ***P* <0.01 *versus* control mono-culture; ^*P* <0.05 and ^^*P* <0.01 *versus* co-culture with ADFs (*n* = 6).

3.3.4.2 Optimal MSC:hepatocyte co-culture ratio

Mitochondrial dehydrogenase activity AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Figure 3.5A). Hepatocytes co-cultured with AT-MSCs had significantly higher MTT activity than control mono-culture from day 1 (1:1 *vs.* 1:2.5 *vs.* 1:5 *vs.* 1:10 *vs.* control, 1.67 ± 0.19 *vs.* 1.75 ± 0.24 *vs.* 1.60 ± 0.20 *vs.* 1.65 ± 0.18 *vs.* 1.39 ± 0.10 OD units; P <0.05) until day 7 (2.65 ± 0.26 *vs.* 3.43 ± 0.24 *vs.* 2.90 ± 0.20 *vs.* 3.05 ± 0.25 *vs.* 1.25 ± 0.15 OD units; P <0.01). Of note, the 1:2.5 co-culture tended to have the highest hepatocyte mitochondrial dehydrogenase activity as compared to the other co-cultures on day 7 of culture.

Cell attachment AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to cell attachment, as compared to control mono-culture (Figure 3.5B). Hepatocytes co-cultured with AT-MSCs had significantly higher SRB activity than control mono-culture from day 1 (3.34 ± 0.20 *vs.* 3.51 ± 0.19 *vs.* 3.38 ± 0.22 *vs.* 3.22 ± 0.15 *vs.* 2.75 ± 0.10 OD units; P <0.05) until day 7 (4.68 ± 0.40 *vs.* 5.58 ± 0.43 *vs.* 5.26 ± 0.50 *vs.* 4.97 ± 0.37 *vs.* 3.00 ± 0.30 OD units; P <0.01). Of note, all co-cultures had similar hepatocyte SRB attachment throughout 7 days of culture.

Albumin secretion AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to albumin secretion, as compared to control mono-culture (Figure 3.5C). Hepatocytes co-cultured with AT-MSCs had significantly higher albumin secretion than control mono-culture from day 3 (2.3 ± 0.3 *vs.* 2.5 ± 0.3 *vs.* 2.3 ± 0.3 *vs.* 2.3 ± 0.2 *vs.* 0.9 ± 0.2 μg/10^6 hepatocytes; P <0.01) until day 7 (4.2 ± 0.4 *vs.* 4.7 ± 0.4 *vs.* 4.6 ± 0.4 *vs.* 4.3 ± 0.3 *vs.* 1.4 ± 0.2 μg/10^6 hepatocytes; P <0.01). Of note, all co-cultures had similar albumin secretion throughout 7 days of culture.

Urea synthesis AT-MSCs co-cultures at all MSC:hepatocyte ratios exhibited a significant hepatotrophic effect, with respect to urea synthesis, as compared to control mono-culture (Figure 3.5D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than control mono-culture from day 1 (21.6 ± 2.0 *vs.* 22.6 ± 2.3 *vs.* 21.8 ± 2.4 *vs.* 21.8 ± 1.6 *vs.* 18.0 ± 2.6 μg/10^6 hepatocytes; P <0.05) until day 7 (35.7 ± 3.0 *vs.* 37.9 ± 3.0 *vs.*34.2 ± 2.9 *vs.* 36.4 ± 2.5 *vs.* 17.0 ± 2.4 μg/10^6 hepatocytes; P <0.01). Of note, all co-cultures had similar albumin secretion throughout 7 days of culture.

Overall AT-MSCs co-culture at the MSC:hepatocyte ratio of 1:2.5 exhibited a significant hepatotrophic effect, as compared to hepatocyte mono-culture, similar to co-cultures at the ratio of 1:1, 1:5, and 1:10. As AT-MSCs reached a 100% confluency at a density of approximately 20,000 cells per cm^2, the ratio of 1:2.5 was used in further experiments to exclude the possibility of MSCs expansion over 7 days of co-culture.

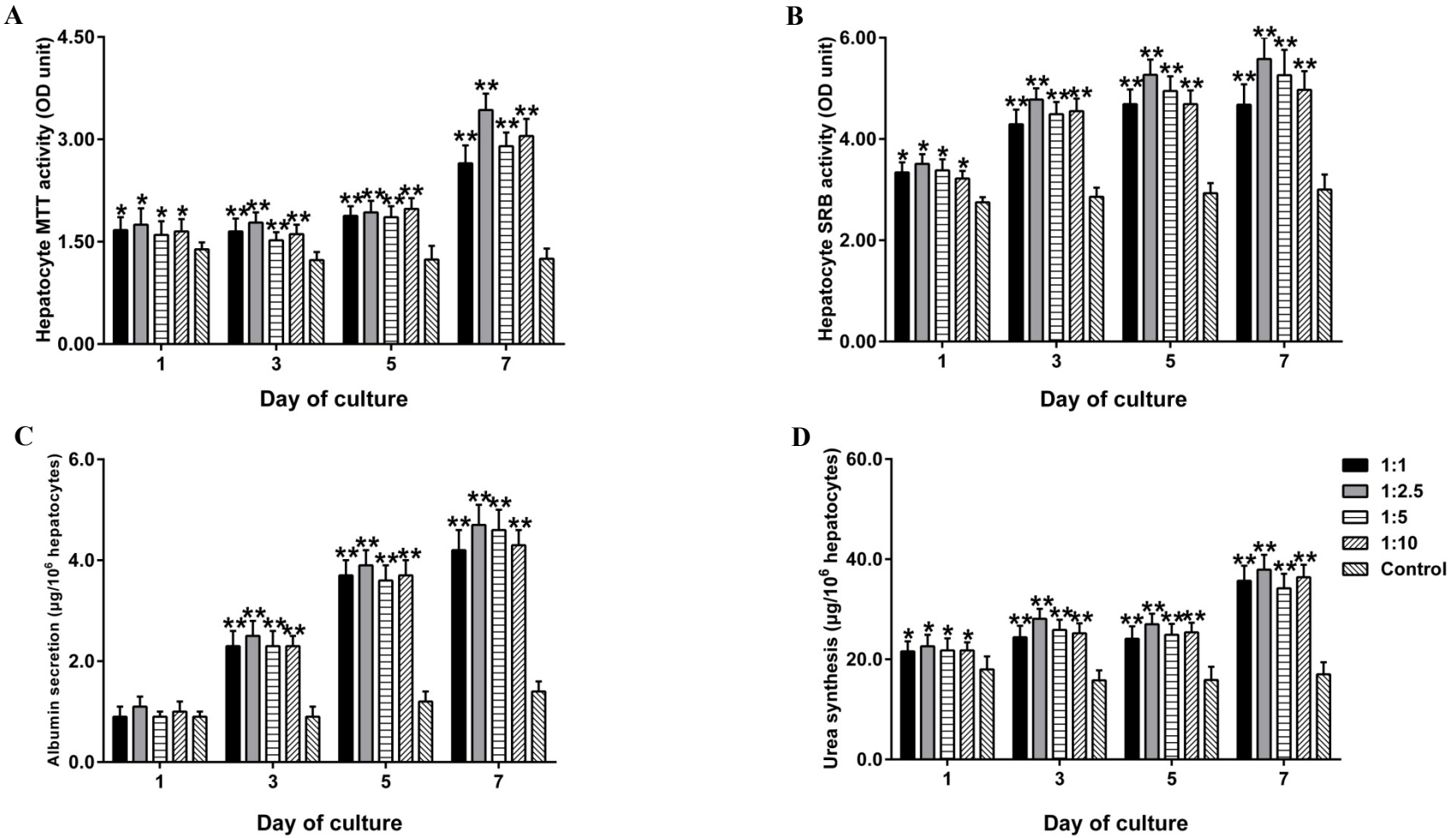

Figure 3.5 MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of hepatocytes co-cultured with AT-MSCs at a predefined MSC:hepatocyte ratio of 1:1, 1:2.5, 1:5, and 1:10 *versus* hepatocyte mono-culture. Co-culture with MSCs at all predefined ratios exhibited significant hepatotrophic effect, to a similar extent among co-cultures. All data were expressed as mean ± SD; **P* <0.05 and ***P* <0.01 *versus* control mono-culture (*n* = 6).

3.3.5 *Hepatotrophic effect of MSCs co-culture on cryopreserved and steatotic hepatocytes*

3.3.5.1 Hepatotrophic effect of AT-MSCs co-culture on steatotic hepatocytes

Mitochondrial dehydrogenase activity AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Figure 3.6A). Moderately steatotic hepatocytes co-cultured with MSCs had significantly higher MTT activity than control mono-culture from day 1 (co-culture *vs.* mono-culture, 1.35 ± 0.20 *vs.* 0.70 ± 0.10 OD units; P <0.01) until day 7 (1.40 ± 0.20 *vs.* 0.60 ± 0.10 OD units; P <0.01).

Cell attachment AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to cell attachment, as compared to control mono-culture (Figure 3.6B). Moderately steatotic hepatocytes co-cultured with MSCs had significantly higher SRB activity than control mono-culture from day 1 (2.25 ± 0.30 *vs.* 1.80 ± 0.15 OD units; P <0.01) until day 7 (2.40 ± 0.39 *vs.* 1.83 ± 0.27 OD units; P <0.01).

Albumin secretion AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to albumin secretion, as compared to control mono-culture (Figure 3.6C). Hepatocytes co-cultured with MSCs had significantly higher albumin secretion than control mono-culture from day 3 (1.2 ± 0.3 *vs.* 0.5 ± 0.1 μg/10^6 hepatocytes; P <0.01) from day 7 (2.4 ± 0.2 *vs.* 0.9 ± 0.1 μg/10^6 hepatocytes; P <0.01).

Urea synthesis AT-MSCs co-culture exhibited a significant trophic effect on moderately steatotic hepatocytes, with respect to urea synthesis, as compared to control mono-culture (Figure 3.6D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than control mono- culture from day 1 (17.6 ± 1.4 *vs.* 9.0 ± 1.0 μg/10^6 hepatocytes; P <0.01) until day 7 (25.4 ± 2.3 *vs.* 8.5 ± 0.9 μg/10^6 hepatocytes; P <0.01).

3.3.5.2 Hepatotrophic effect of AT-MSCs co-culture on cryopreserved hepatocytes

Mitochondrial dehydrogenase activity AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Figure 3.7A). Moderately steatotic hepatocytes co-cultured with AT-MSCs had significantly higher MTT activity than control mono-culture from day 1 (co-culture *vs.* mono-culture, 1.12 ± 0.14 *vs.* 0.68 ± 0.10 OD units; P <0.01) until day 7 (1.44 ± 0.21 *vs.* 0.54 ± 0.11 OD units; P <0.01).

Cell attachment AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to cell attachment, as compared to control mono-culture (Figure 3.7B). Moderately steatotic hepatocytes co-cultured with MSCs had significantly higher SRB activity than control mono-culture from day 1 (2.59 ± 0.33 *vs.* 1.80 ± 0.19 OD units; P <0.01) until day 7 (2.75 ± 0.41 *vs.* 2.24 ± 0.32 OD units; P <0.01).

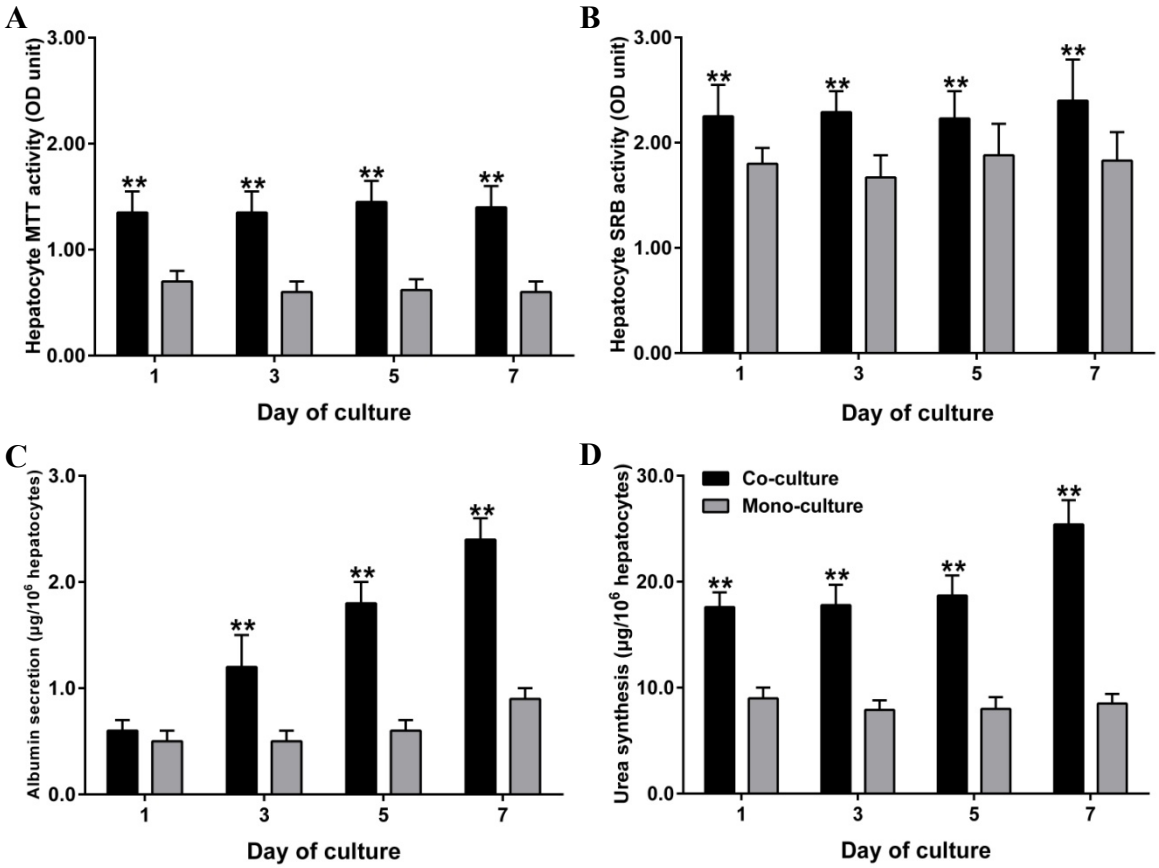

Figure 3.6 **MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of moderately steatotic hepatocytes co-cultured with AT-MSCs at a ratio of 2.5:1 *versus* hepatocyte mono-culture.** Co-culture with MSCs had significant trophic effect on steatotic hepatocytes within 7 days of *in vitro* culture. All data were expressed as mean ± SD (error bar); **P* <0.05 and ***P* <0.01 *versus* control mono-culture (*n* = 6).

Albumin secretion AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to albumin secretion, as compared to control mono-culture (Figure 3.7C). Hepatocytes co-cultured with AT-MSCs had significantly higher albumin secretion than control mono-culture from day 1 (0.7 ± 0.1 *vs.* 0.5 ± 0.1 µg/10^6 hepatocytes; *P* <0.01) until day 7 (2.1 ± 0.3 *vs.* 0.9 ± 0.1 µg/10^6 hepatocytes; *P* <0.01).

Urea synthesis AT-MSCs co-culture exhibited a significant trophic effect on cryopreserved hepatocytes, with respect to urea synthesis, as compared to control mono-culture (Figure 3.7D). Hepatocytes co-cultured with MSCs had significantly higher urea synthesis than control mono-culture from day 1 (13.0 ± 1.6 *vs.* 8.8 ± 1.3 µg/10^6 hepatocytes; *P* <0.01) until day 7 (19.9 ± 2.3 *vs.* 8.4 ± 1.1 µg/10^6 hepatocytes; *P* <0.01).

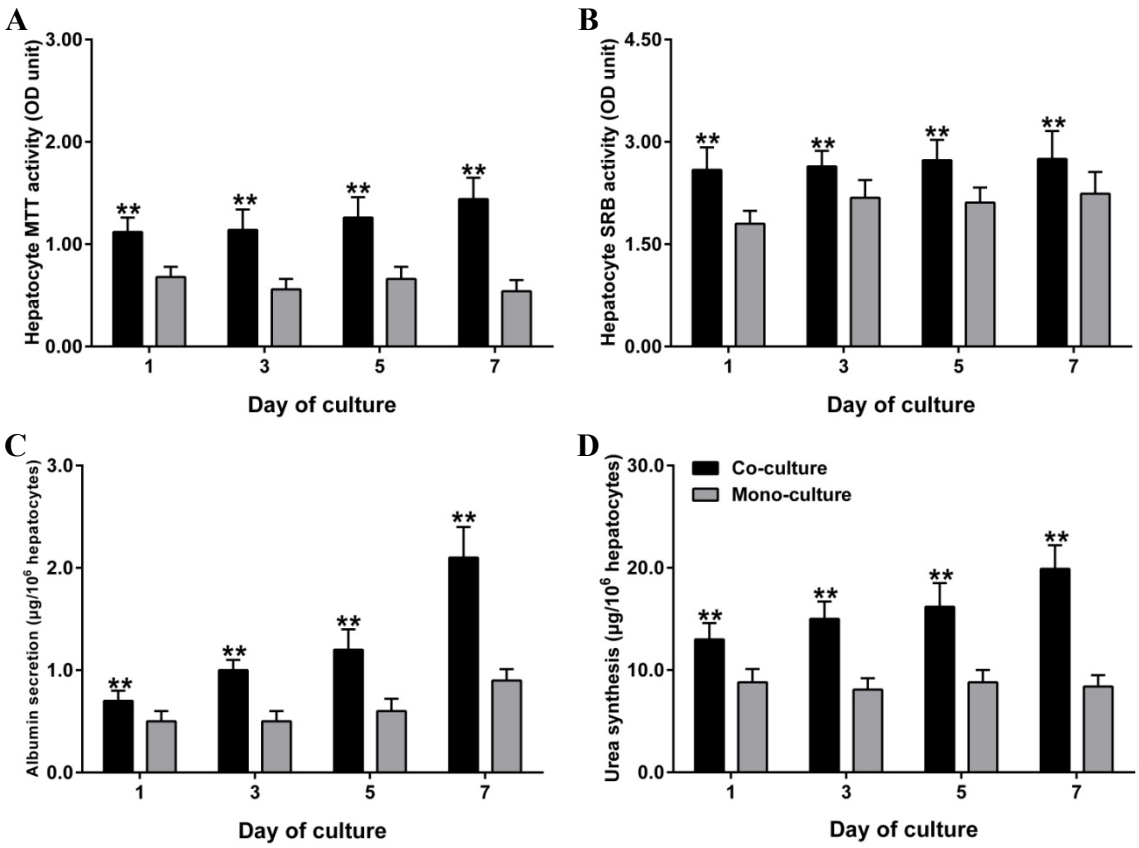

Figure 3.7 MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of cryopreserved hepatocytes co-cultured with AT-MSCs at a ratio of 2.5:1 *versus* hepatocyte mono-culture. Co-culture with MSCs had significant trophic effect on cryopreserved hepatocytes within 7 days of *in vitro* culture. All data were expressed as mean ± SD (error bar); **P* <0.05 and ***P* <0.01 *versus* control mono-culture (*n* = 6).

3.3.6 *Contribution of soluble factors to hepatotrophic effect of MSCs co-culture*

3.3.6.1 Soluble factors alone contribute minimally to hepatotrophic effect of MSCs co-culture

Mitochondrial dehydrogenase activity MSCs indirect co-culture using Transwell plates exhibited no significant hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture (Table 3.3). Mitochondrial dehydrogenase activity remained similar between indirect co-culture and control mono-culture from day 1.

Cell attachment MSCs indirect co-culture exhibited no significant hepatotrophic effect, with respect to hepatocyte attachment, as compared to control mono-culture (Table 3.3). SRB cell attachment remained similar between indirect co-culture and control mono-culture from day 1 until day 7.

Table 3.3 MTT activity and SRB cell attachment (mean ± SD) of hepatocytes indirectly co-cultured with AT-MSCs on Transwell plates *versus* mono-cultured hepatocytes

	MTT (OD unit)				SRB (OD unit)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
iCo-	1.60 ± 0.16	1.30 ± 0.10	1.25 ± 0.10	1.31± 0.09	2.88 ± 0.20	3.10 ± 0.20	3.01 ± 0.16	3.05 ± 0.21
Mono-	1.59 ± 0.18	1.25 ± 0.16	1.27 ± 0.18	1.29 ± 0.09	2.80 ± 0.14	2.91 ± 0.17	2.94 ± 0.15	2.99 ± 0.14

iCo-, indirect co-culture; Mono-, mono-culture.

Albumin secretion MSCs indirect co-culture exhibited no significant hepatotrophic effect, with respect to albumin secretion, as compared to control mono-culture (Table 3.4). Albumin secretion was similar between indirect co-culture and control mono-culture from day 1 until day 7.

Urea synthesis MSCs indirect co-culture exhibited no significant hepatotrophic effect, with respect to urea synthesis, as compared to control (Table 3.4). Urea synthesis was similar between indirect co-culture and control mono-culture from day 1 until day 7.

Table 3.4 Albumin secretion and urea synthesis (mean ± SD) of hepatocytes indirectly co-cultured with AT-MSCs on Transwell plates *versus* mono-cultured hepatocytes

	Albumin (μg/10^6 hepatocytes)				Urea (μg/10^6 hepatocytes)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
iCo-	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	18.6 ± 2.4	16.9 ± 2.5	17.1 ± 2.4	15.6 ± 2.0
Mono-	1.0 ± 0.1	0.9 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	19.0 ± 2.3	17.5 ± 2.3	16.7 ± 2.8	17.0 ± 3.0

iCo-, indirect co-culture; Mono-, mono-culture.

3.3.6.2 MSCs co-culture CM have minimal hepatotrophic effect

Mitochondrial dehydrogenase activity MSCs co-culture CM exhibited a limited hepatotrophic effect, with respect to mitochondrial dehydrogenase activity, as compared to control mono-culture throughout 7 days of indirect co-culture (Table 3.5). Hepatocytes cultured with MSCs co-culture CM had significantly higher MTT activity than mono-cultured hepatocytes (CM culture *vs.* mono-culture, 1.58 ± 0.15 *vs.* 1.39 ± 0.14 OD units; $P < 0.01$) on day 1; however, mitochondrial dehydrogenase activity remained similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 3 until day 7.

Cell attachment MSCs co-culture CM exhibited no significant hepatotrophic effect, with respect to hepatocyte attachment, as compared to control mono-culture (Table 3.5). SRB cell attachment remained similar between hepatocyte cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7.

Table 3.5 MTT activity and SRB cell attachment (mean ± SD) of hepatocytes cultured with AT-MSCs co-culture CM *versus* mono-cultured hepatocytes

	MTT (OD unit)				SRB (OD unit)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
Cond	1.58 ± 0.15**	1.21 ± 0.19	1.25 ± 0.10	1.29 ± 0.17	2.71 ± 0.26	2.91 ± 0.24	2.93 ± 0.26	2.97 ± 0.24
Mono-	1.39 ± 0.14	1.23 ± 0.15	1.24 ± 0.16	1.25 ± 0.18	2.75 ± 0.27	2.86 ± 0.23	2.93 ± 0.25	3.00 ± 0.22

Cond, conditioned; Mono-, mono-culture; **$P < 0.01$ *versus* mono-culture.

Albumin secretion No albumin secretion was detected in MSCs mono-culture. MSCs co-culture CM exhibited no significant hepatotrophic effect, with respect to albumin secretion, as compared to control mono-culture (Table 3.6). Albumin secretion remained similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7.

Urea synthesis MSCs co-culture CM exhibited no significant hepatotrophic effect, with respect to urea synthesis, as compared to control mono-culture (Table 3.6). Urea synthesis was similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7.

Table 3.6 Albumin secretion and urea synthesis (mean ± SD) of hepatocytes cultured with AT-MSCs co-culture CM *versus* mono-cultured hepatocytes

	Albumin (μg/10^6 hepatocytes)				Urea (μg/10^6 hepatocytes)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
Cond	1.0 ± 0.1	1.0 ± 0.3	1.1 ± 0.2	1.2 ± 0.1	19.0 ± 2.6	15.6 ± 2.6	16.4 ± 2.3	15.6 ± 2.6
Mono-	0.9 ± 0.1	0.9 ± 0.2	1.2 ± 0.3	1.4 ± 0.2	18.0 ± 2.6	15.8 ± 2.8	15.9 ± 1.9	17.0 ± 2.0

Cond, conditioned; Mono-, mono-culture.

3.3.7 *Anti-apoptotic effect of MSCs co-culture on hepatocytes*

3.3.7.1 MSCs co-culture suppresses caspase-mediated spontaneous hepatocyte apoptosis

MSCs direct co-culture No CCK18 or CK18 release was detected in MSCs mono-culture. Direct co-culture with MSCs significantly reduced CCK18 release from hepatocytes as compared to control mono-culture (Figure 3.8A). Soluble CCK18 level remained significantly lower in hepatocytes co-cultured with MSCs than that in mono-cultured hepatocytes from day 1 (co-culture *vs.* mono-culture, 18.5 ± 1.4 *vs.* 23.6 ± 1.5 U/10^6 hepatocytes; P <0.01) until day 7 (0.3 ± 0.1 *vs.* 1.6 ± 0.1 U/10^6 hepatocytes; P <0.01). Direct co-culture with MSCs also significantly reduced CK18 release from hepatocytes, as compared to hepatocyte mono-culture (Figure 3.8B), from day 1 (91.2 ± 8.2 *vs.* 123.6 ± 16.3 U/10^6 hepatocytes; P<0.01) until day 7 (20.0 ± 1.9 *vs.* 52.0 ± 5.0 U/10^6 hepatocytes; P <0.01). As is shown in Figure 3.8C, CCK18/CK18 ratio (cell death mode; Figure 3.8C) remained similar between co-cultured hepatocytes and mono-cultured hepatocytes from day 1 (20.2% ± 3.0% *vs.* 19.1% ± 2.6%; P >0.05) until day 7 (1.5% ± 0.1% *vs.* 3.0% ± 0.4%; P >0.05).

MSCs indirect co-culture using Transwell plates Indirect co-culture with MSCs had no significant effect on hepatocyte CCK18 or CK18 release as compared to control mono-culture (Table 3.7). Soluble CCK18 level remained similar between hepatocytes indirectly co-cultured with MSCs and mono-cultured hepatocytes from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes indirectly co-cultured with MSCs and mono-cultured hepatocytes from day 1

until day 7. CCK18/CK18 ratio remained similar between hepatocytes indirectly co-cultured with MSCs and mono-cultured hepatocytes (Table 3.4) from day 1 until day 7.

MSCs co-culture CM MSCs co-culture CM had no significant effect on hepatocyte CCK18 or CK18 release from s as compared to control mono-culture (Table 3.8). Soluble CCK18 level remained similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes cultured with MSCs co-culture CM and mono-cultured hepatocytes from day 1 until day 7. CCK18/CK18 ratio remained similar between hepatocytes cultured with MSCs co-culture CM and hepatocyte mono-culture (Table 3.7) from day 1 until day 7.

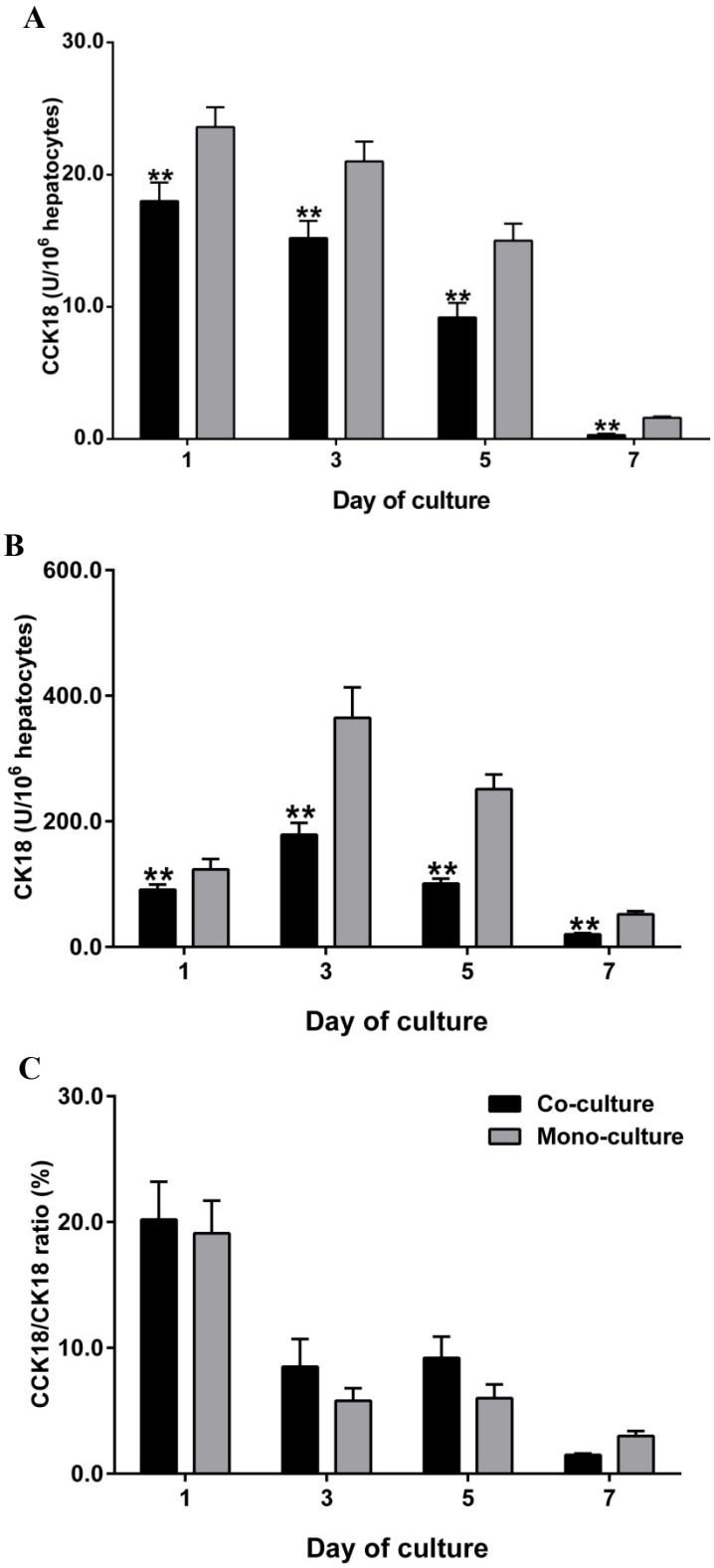

Figure 3.8 CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes cultured with AT-MSCs *versus* mono-cultured hepatocytes. MSCs co-culture significantly reduced caspase-mediated apoptosis and total death of hepatocytes without altering cell death mode within 7 days of *in vitro* culture. All data were expressed as mean ± SD; ***P* <0.01 *versus* control mono-culture (*n* = 6).

Table 3.7 **CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes indirectly co-cultured with MSCs *versus* mono-cultured hepatocytes**

	CCK18 (U/10^6 hepatocytes)				CK18 (U/10^6 hepatocytes)				CCK18/CK18 ratio (%)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
iCo-	20.8 ± 2.4	23.2 ± 2.3	15.9 ± 1.7	1.6 ± 0.2	108.8 ± 12.4	390.0 ± 35.0	276.0 ± 33.0	50.4 ± 4.6	19.1 ± 2.8	5.9 ± 1.1	5.8 ± 1.3	3.2 ± 0.1
Mono-	23.6 ± 2.5	21.0 ± 2.5	15.7 ± 1.5	1.6 ± 0.2	123.6 ± 16.4	352.0 ± 39.0	259.5 ± 35.0	52.0 ± 4.0	19.1 ± 2.5	8.2 ± 1.3	6.1 ± 1.1	3.1 ± 0.3

iCo-, indirect co-culture; Mono-, mono-culture.

Table 3.8 **CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes cultured with MSCs co-culture CM *versus* mono-cultured hepatocytes**

	CCK18 (U/10^6 hepatocytes)				CK18 (U/10^6 hepatocytes)				CCK18/CK18 ratio (%)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
iCo-	20.3 ± 2.1	22.5 ± 2.0	16.3 ± 1.8	2.0 ± 0.4	110.5 ± 12.5	360.0 ± 33.3	286.0 ± 31.7	54.1 ± 4.0	18.4 ± 2.0	6.3 ± 1.1	5.7 ± 1.0	3.7 ± 0.2
Mono-	23.8 ± 2.6	21.0 ± 2.4	15.9 ± 2.0	2.1 ± 0.3	123.5 ± 14.0	358.0 ± 40.0	266.7 ± 33.0	54.1 ± 4.3	19.3 ± 2.4	5.9 ± 1.2	6.0 ± 1.1	3.9 ± 0.2

Cond, conditioned; Mono-, mono-culture.

3.3.7.2 MSCs co-culture protects hepatocytes from staurosporine-induced cell apoptosis

Effect of staurosporine on hepatocytes Addition of 0.5-μM staurosporine had no significant effect on CCK18 release from hepatocytes as compared to blank control; however, the presence of 1-, 2.5-, 5-, and 10-μM staurosporine significantly increased soluble CCK18 level as compared to that of 0.5-μM staurosporine (0.5 μM *vs.* 1 μM *vs.* 2.5 μM *vs.* 5 μM *vs.* 10 μM, 24.7 ± 2.9 *vs.* 44.7 ± 3.6 *vs.* 51.2 ± 5.0 *vs.* 56.1 ± 5.5 *vs.* 70.2 ± 5.7 U/10^6 hepatocytes; P <0.01; Figure 3.9A) in a dose-dependent manner. Similarly, addition of 0.5-μM staurosporine had no significant effect on CK18 release from hepatocytes as compared to that of 0-μM staurosporine; however, in the presence of 1-, 2.5-, 5-, and 10-μM staurosporine significantly increased soluble CK18 level as compared to that at 0.5-μM staurosporine (136.1 ± 14.8 *vs.* 156.9 ± 18.9 *vs.*176.0 ± 20.4 *vs.* 187.3 ± 25.0 *vs.* 204.8 ± 17.9 U/10^6 hepatocytes, P <0.01; Figure 3.9B) in a dose-dependent manner. Moreover, addition of 1-, 2.5-, 5-, and 10-μM staurosporine switched cell death mode of hepatocytes from necrosis (lower CCK18/CK18 ratio) to apoptosis (higher CCK18/CK18 ratio) as shown in Figure 3.9C.

Effect of MSCs co-culture on staurosporine-induced hepatocyte apoptosis Hepatocytes co-cultured with or without MSCs were subjected to 1-μM staurosporine to investigate whether MSCs co-culture could protect hepatocytes from chemically-induced apoptosis. As is shown in Figure 3.10A, co-culture with MSCs significantly reduced staurosporine-induced hepatocyte apoptosis (soluble CCK18 level) as compared to hepatocyte mono-culture: co-culture *vs.* mono-culture *vs.* blank, 26.5 ± 2.5 *vs.* 44.7 ± 6.3 *vs.* 21.2 ± 2.1 U/10^6 hepatocytes (P <0.01). However, MSCs co-culture had no significant effect on staurosporine-induced total cell death (soluble CK18 level) of hepatocytes (Figure 3.10B): 148.0 ± 10.7 *vs.* 156.9 ± 18.9 *vs.* 111.3 ± 17.0 U/10^6 hepatocytes (P <0.01). Moreover, MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis switch of hepatocyte death mode (Figure 3.10C): CCK18/CK18 ratio, 18.0% ± 2.2% *vs.* 28.6% ± 2.4% *vs.* 18.9% ± 2.6% (P <0.05).

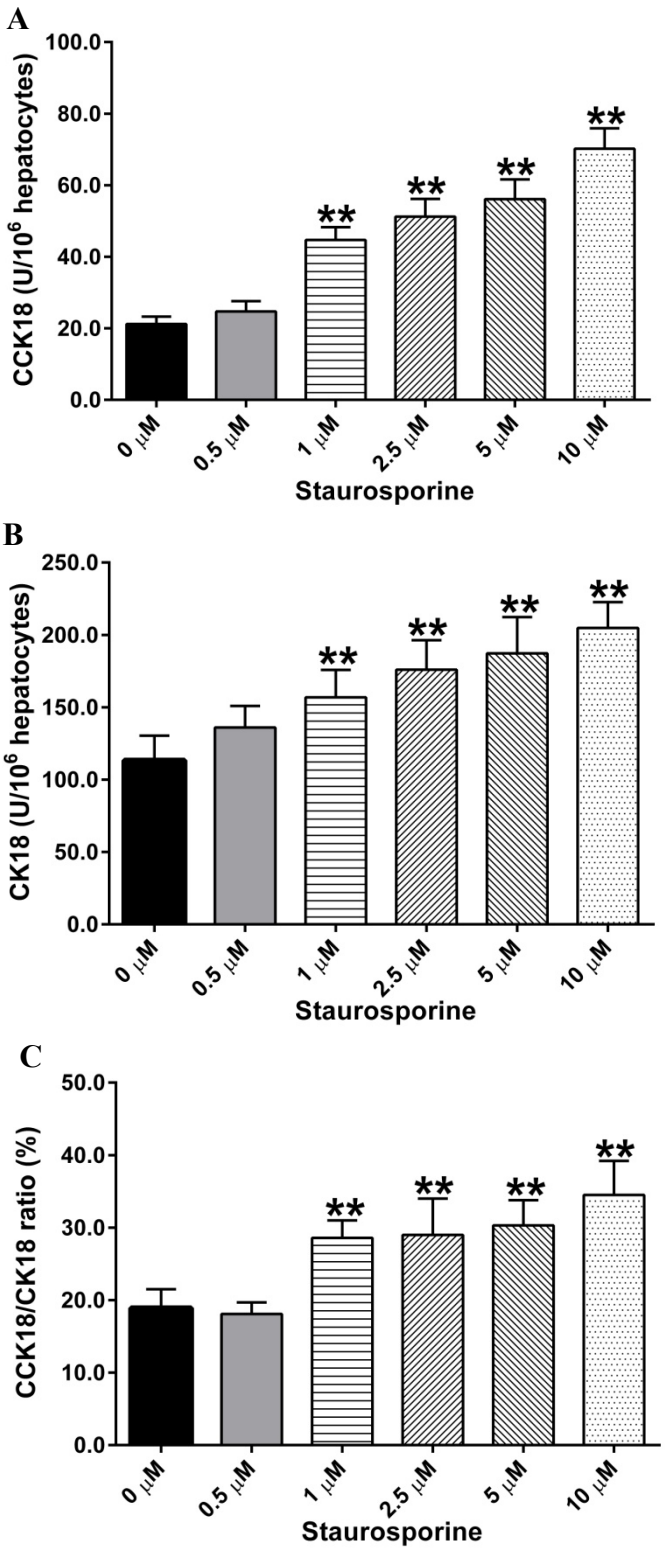

Figure 3.9 CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes subjected to 0-, 0.5-, 1-, 2.5-, 5-, 10-μM staurosporine. Addition of 0.5-μM staurosporine had no significant effect on apoptosis, total death, and death mode of hepatocytes, while addition of 1-, 2.5-, 5-, and 10-μM staurosporine significantly increased apoptosis and total death of hepatocytes in a dose-dependent manner and switched cell death mode of hepatocytes from necrosis to apoptosis. All data were expressed as mean ± SD; $^{**}P < 0.01$ *versus* control mono-culture ($n = 6$).

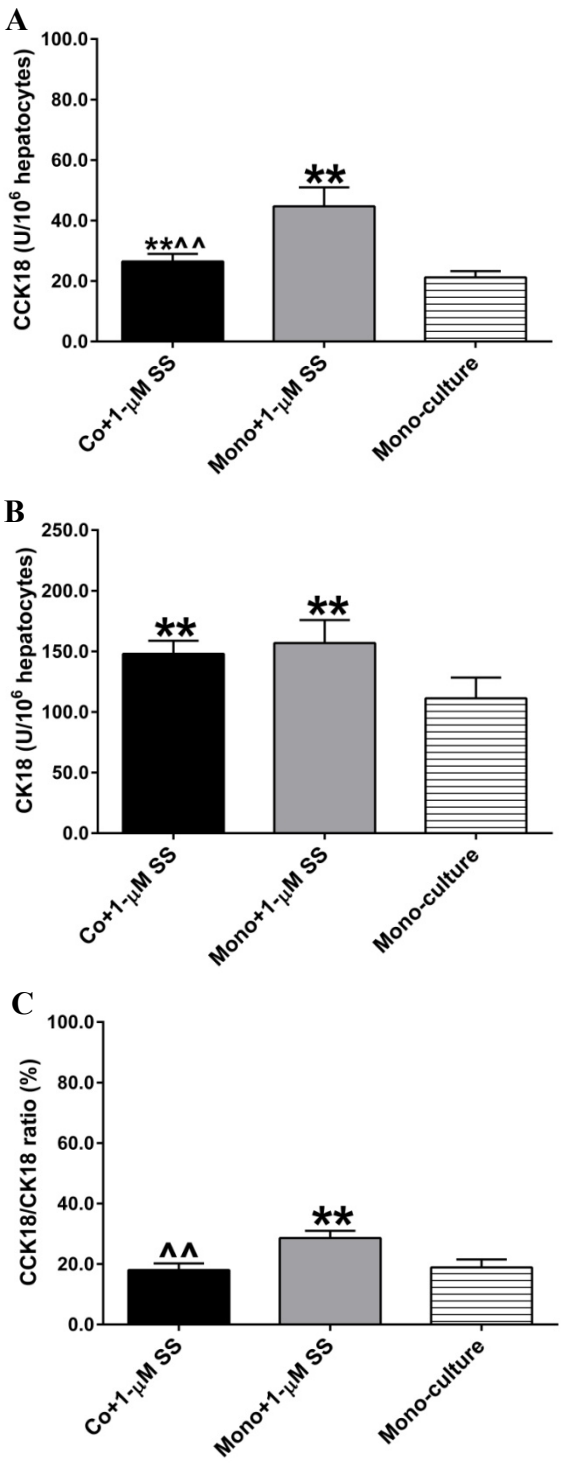

Figure 3.10 CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes subjected to 1-μM staurosporine and co-cultured with or without AT-MSCs *versus* hepatocyte mono-culture treated without staurosporine. Addition of staurosporine significantly increased caspase-mediated apoptosis and total death of hepatocytes and switched death mode of hepatocytes from necrosis to apoptosis, while MSCs co-culture significantly reduced staurosporine-induced hepatocyte apoptosis and reversed death mode switch. All data were expressed as mean ± SD; $^{**}P < 0.01$ *versus* non-staurosporine-treated, mono-cultured hepatocytes; $^{\wedge\wedge}P < 0.01$ *versus* staurosporine-treated, mono-cultured hepatocytes ($n = 6$). Co, hepatocyte co-culture; mono, hepatocyte mono-culture; SS, staurosporine.

3.4 Discussion

3.4.1 Potentials of liver NPCs in HCT

Multiple techniques have been reported to separate liver NPCs from parenchymal cells (hepatocytes), among which selective enzymatic digestion and unit gravity sedimentation is most frequently used, due to the fact that NPCs are in a relatively small size. Doolittle *et al.* (1987) identified and characterised an unfractioned population of liver NPCs by flow cytometry and reaffirmed that cell size was linearly correlated to the sedimentation velocity. Primary liver NPCs were successfully isolated in the present work using the modified protocol as previously reported (Najimi *et al.*, 2007); the purity of liver NPCs was over 90% as determined on cell morphology following the first culture medium refreshment.

NPCs occupy only 6.5% of the total volume of the liver but account for approximately 40% of the total number of liver cells. Liver NPCs themselves are highly heterogeneous, in terms of cell morphology, functionality, and topography, consisting of sinusoidal hepatic endothelial cells, Kupffer cells, and hepatic stellate cells lining the liver sinusoid, as well as ‘‘pit cells’’, namely, liver residing lymphocytes. Liver NPCs play an active role in regulating biological activities of hepatocytes *in vivo*, including hepatocyte survival, proliferation, maintenance, and metabolism. In addition, liver NPCs are also involved in inflammatory and immune responses of the liver in the setting of sepsis (Scott *et al.*, 2005), malignancy, autoimmune disorder, and immune rejection (Chen *et al.*, 2002).

Liver NPCs show a significant trophic effect on hepatocytes co-cultured *in vitro*. Sinusoidal endothelial cells and Kupffer cells regulate hepatocyte secretion of albumin and alpha 1-acid glycoprotein by monokines (especially IL-6), dexamethasone, and inducible nitric oxide synthase pathway (Itoh *et al.*, 1994). In a study regarding bioartificial liver system, co-cultivation of hepatocytes with NPCs significantly multiplied amino acid conversion, lactate production, nitrogen metabolism, drug detoxicification, and clearance of aggregated gamma-globulin as compared to hepatocytes alone (Nedredal *et al.*, 2007). The present work showed that co-culture of hepatocytes with autologous liver NPCs even at a very low NPC:hepatocyte ratio (1:10) was still beneficial with respect to overall cellular viability, attachment, and liver-specific metabolic function. It suggests a possibility that co-transplantation of autologous liver NPCs can improve current HCT practice. An additional benefit of co-transplanting NPCs is to protect parenchymal cells from immune elimination. Yang *et al.* (2009) reported that hepatic stellate cells had a profound inhibitory effect on T-cells and effectively prevented islet allografts from acute rejection in mice. These comprehensive T-cell inhibitory activities included induction of apoptosis of graft-infiltrating antigen-specific effector T-cells and marked expansion of CD4(+) Forkhead box protein 3(+) T-regulatory cells by interferon-gamma signalling (Yang *et al.*, 2009).

However, the use of co-culture of hepatocytes with liver NPCs is primarily limited by the high intrinsic heterogeneity (Pan *et al.*, 1996) and possible fibrogenic potential of NPCs in the presence of

profibrotic factors (Perepelyuk *et al.*, 2013). These limitations result in unfavourable reproducibility and safety profile of NPCs co-culture. The present work showed that expansion potential of primary liver NPCs isolated from adult donors was very limited as the great majority of this heterogeneous cell population is terminally differentiated. Moreover, cellular components and constituents remained unevaluated among previous studies regarding co-culture of hepatocytes with liver NPCs. A study regarding fractioning and characterising liver NPCs by immunophenotyping is ongoing in our research group.

3.4.2 Hepatotrophic effect of MSCs co-culture on human hepatocytes

3.4.2.1 Optimisation of cellular source and ratio for MSCs co-culture

In vitro hepatotrophic effect of MSCs co-culture has been well documented in current literature. Gu and his colleagues (2009[a]; 2009[b]; 2009[c]) isolated BM-MSCs from pigs and preserved the morphology and liver-specific metabolic function of porcine hepatocytes in a three-dimensional co-culture system. Fitzpatrick *et al.* (Cell Transplant, Epub ahead of print) also co-cultured human hepatocytes with human UC matrix-derived MSCs and successfully improved viability and functionality of co-cultured hepatocytes. Whether AT-MSCs has comparable trophic effect on co-cultured hepatocytes remains occasionally studied as AT-MSCs are a new family member of MSCs. No da *et al.* (2012) co-cultured human hepatocytes isolated from partial hepatectomy specimen and human AT-MSCs at a ratio of 1:1 to generate hepatocyte spheroids and improved albumin secretion and CYP450 activity of co-cultured hepatocytes over one week. Chen *et al.* (2012) isolated AT-MSCs from human orbital fat and co-cultured these MSCs with rat hepatocytes; this co-culture protected rat hepatocytes from ALF serum by upregulating immunomodulatory IL-6. Saito *et al.* (2013) reported that AT-MSCs transplantation attenuated ischaemia/reperfusion-induced liver injury and promoted liver regeneration as early as 6 hours after reperfusion by upregulating VEGF expression. The present work demonstrated for the first time that human AT-MSCs had a significant trophic effect on co-cultured human hepatocytes, to an extent similar to BM- and UC-MSCs. As AT-MSCs are more readily available and subject to less ethical controversies, AT-MSCs are determined to be the optimal MSCs for hepatocyte co-culture.

The present work also showed that human MSCs co-culture improved hepatocyte cellular activity and liver-specific metabolic function to a greater extent than human ADFs co-culture. NIH/3T3 cells, a standardised fibroblast cell line, are frequently used as the ‘‘feeder cells’’ for co-cultured hepatocytes in bioartificial liver and *in vitro* drug hepatotoxicity screening systems (Cho *et al.*, 2007; Chan *et al.*, 2013). Devitalised fibroblasts and fibroblast CM alone were reported to be minimally hepatotrophic *in vitro* (Bhandari *et al.*, 2001). Hepatotrophic effect of fibroblast co-culture is believed to synergistically result from homotypic hepatocyte-to-hepatocyte interaction, heterotypic hepatocyte-to-fibroblast communication, hepatocyte-to-ECM contact, and soluble factors. Therefore, greater hepatotrophic effect of MSCs co-culture may be derived from potentiated synergistic effect of these factors rather than as adherent matrix or source of paracrine factors alone.

Theoretically a small number of MSCs should exhibit a significant trophic effect on co-cultured hepatocytes *in vitro* as liver NPCs occupy only a small portion of liver cells. Gu *et al.* (2009[c]) randomly co-cultured hepatocytes and BM-MSCs and predefined MSC:hepatocyte ratio at 1:1, 1:2, 1:5, and 1:10, respectively, with the total cell number fixed at 1 million per well; the optimal liver-specific metabolic function was achieved in the co-culture at the ratio of 1:2, with respect to albumin secretion and urea synthesis, which reached the peak on day 2 of co-culture. Fitzpatrick *et al.* (Cell Transplant, Epub ahead of print) modified the co-culture protocol of hepatocytes with UC-MSCs (plating of hepatocytes followed by that of MSCs) and predefined MSC:hepatocyte ratio, with the number of hepatocytes fixed at 150,000/cm^2, at 1:3, 1:6, and 1:10, respectively; UC-MSCs co-culture exhibited a similar hepatotrophic effect, regardless of MSC:hepatocyte ratio, within 7 days of *in vitro* culture with respect to albumin secretion and urea synthesis. This similarity between low- and high-density UC-MSCs co-culture may be attributed to the fact that UC-MSCs are highly proliferative *in vitro* and able to reach the confluency within 7 days of culture even starting from a low seeding density. In contrast, the co-culture protocol was further modified in the present work, by seeding hepatocytes onto pre-existing AT-MSCs monolayer in a well-organised, three-dimensional manner, with MSC:hepatocyte ratio predefined, with the number of hepatocytes fixed at 50,000/cm^2, at 1:1, 1:2.5, 1:5, and 1:10, respectively. The present work showed that even a very low seeding density of AT-MSCs (5,000/cm^2) still had trophic effect on co-cultured hepatocytes generally similar to the high-density AT-MSCs (50,000/cm^2). This similarity between low- and high-density AT-MSCs may result from the fact that AT-MSCs have a relatively large cell size compared to BM- and UC-MSCs and provide adequate cellular and matrical surface even at a low seeding density. AT-MSCs normally reach 100% confluency at a density of 25,000/cm^2; therefore, the optimal MSC:hepatocyte ratio was determined to be 1:2.5, equalling a 20,000/cm^2 seeding density of MSCs, at which AT-MSCs would become 100% confluent within a very short time, to exclude the possible confounding effect of MSCs proliferation *in vitro* throughout co-culture.

3.4.2.2 Hepatotrophic effect of MSCs co-culture on steatotic hepatocytes

A seriously steatotic donor liver rejected for the use of OLT will normally also fail isolation of hepatocytes. The paradox arising from availability of donor liver is that only poor-quality, usually steatotic, donor liver tissues will be assigned for HCT use in most cases. Furthermore, isolated steatotic hepatocytes normally exhibit a poor metabolic function and susceptibility to cellular injury *in vitro*, although a steatohepatitis patient usually exhibit no abnormality in liver gross morphology and metabolic function (Donato *et al.*, 2006; Donato *et al.*, 2007). Compromise of steatotic hepatocytes may be compensated by the huge liver function reserve and a high-level hepatocyte self-renewal and turnover, while steatotic hepatocytes have already shown morphological distortion and molecular dysregulation. Compensatory hepatic progenitor cell expansion accompanies steatotic hepatocyte replicative arrest (Cho *et al.*, 2010), and steatotic liver is thought to be a suitable source for isolation of hepatic progenitor cells (Tolosa *et al.*, 2011).

A comparative genomic profiling analysis in obese insulin-resistant Zucker rats with

spontaneous liver steatosis demonstrated that dysregulated expression of metabolic and survival genes, including defence/acute phase-, detoxification-, cell growth/proliferation-, and protein synthesis/transformation-associated genes might lead to the vulnerability of steatotic hepatocytes to cell injury (Buqué *et al.*, 2010), e.g., hypoxia-reoxygenation injury, which could be reversed by defatting (Berthiaume *et al.*, 2009) and mitochondrial uncoupling protein-2 blockade (Evans *et al.*, 2012). Activation of TGF-β signalling and production of ROS are reported to contribute to hepatocyte death and lipid accumulation in non-alcoholic steatohepatitis (Yang *et al.*, 2013) and also sensitise hepatocyte susceptibility to acute toxic effect of acetaminophen (Kučera *et al.*, 2012) and proapoptotic effect of TNF-α (Zhang *et al.*, 2010).

Hayashi *et al.* (2007) reported an interesting rat steatotic HCT study: steatotic hepatocytes secreted significantly less albumin *in vitro* than nonsteatotic counterparts; however, intrasplenically transplanted steatotic hepatocytes produced a serum albumin level similar to nonsteatotic cells. It is possible that *in vivo* factors, such as growth factors and soluble cytokines, improve steatotic hepatocyte metabolic function by modifying and improving cellular biological and molecular activities. Sun *et al.* (2003) showed that IL-6 pretreatment prevented sinusoidal endothelial cell death in response to ischaemia/reperfusion injury, activated cell survival signal transducer and activator of transcription factor 3 in both hepatocytes and sinusoidal endothelial cells, and also improved steatotic liver isograft microcirculation. Hong *et al.* (2004) further demonstrated that protective effect of IL-6 treatment on steatotic hepatocytes *in vivo* resulted from increased mitochondrial β oxidation of fatty acid and export of triglyceride and cholesterol as mediated by peroxisome proliferator-activated receptor α and TNF-α signalling pathways. The present work reported for the first time that MSCs co-culture could also improve cellular activity and liver-specific metabolic function of steatotic hepatocytes as for nonsteatotic hepatocytes. The effect size of MSCs co-culture was similar between steatotic hepatocytes and nonsteatotic hepatocytes as compared to mono-cultured counterparts, with respect to improvement in liver-specific metabolism. As reported by Gu and his colleagues (2009[a]), MSCs secreted a massive amount of IL-6 into hepatocyte co-culture system, and neutralization of IL-6 compromised hepatotrophic effect of MSCs co-culture with respect to albumin secretion and urea synthesis. The role of other *in vivo* factors, such other cytokines, ECM, and heterotypic hepatocyte-to-NPC interaction, in trophic effect of MSCs co-culture on steatotic hepatocytes remains to be investigated. A possible mechanism is that MSCs expedite ROS elimination in co-cultured hepatocytes as human BM-MSCs was reported to inhibit production of ROS in co-cultured macrophages in a dose-dependent manner by a paracrine mechanism (Tsyb *et al.*, 2008)

3.4.2.3 Hepatotrophic effect of MSCs co-culture on cryopreserved hepatocytes

Cryopreservation was thought to be capable of preserving and maintaining the ultrastructural characteristics and metabolic, biochemical and toxicological functions of hepatocytes to some extent. However, cryopreserved hepatocytes are inevitably subject to loss of viability and impairment in metabolic function following thawing and plating. This freezing/thawing-associated injury results

from the underlying cellular and molecular changes. Rijntjes *et al.* (1986) reported that formation of more 'blebs', lipid droplets, and lysosomes were observed in cryopreserved hepatocytes after thawing, accompanied by increased lactate dehydrogenase release and decreased albumin secretion. This ultrastructural distortion was primarily attributed to the change in cytoplasmic osmolality and subsequent intracellular ice crystal formation. Dou *et al.* (1992) also recommended that at least a 38-hour recovery time after seeding should be given for thawed human hepatocytes to stabilise cellular membrane integrity, protein synthesis, and drug metabolism enzymes. Interestingly, profound dysregulation of gene expression, invovled in liver-specific metabolism, cellular apoptosis, and proteasomal protein recycling, was observed in hepatocytes after plating rather than after thawing (Richert *et al.*, 2006). Oxidative stress manifesting as intracellular accumulation of ROS was thought to partially contribute to this gene expression dysregulation (Stevenson *et al.*, 2007).

A major problem associated with thawing of cryopreserved adult human hepatocytes is poor attachment efficiency and rapid reduction in cellular survival. A lot of work has been done to improve viability and metabolic function of cryopreserved hepatocytes by modifying cryopreservation protocols and cryoprotectants. Birraux *et al.* (2002) reported that three-dimensional cryopreservation of rat hepatocytes sandwiched between two layers of collagen type I could adequately restore cellular and liver-specific metabolic function of hepatocytes. Lee *et al.* (2012) also reported that cryopreservation of spheroid hepatocytes was more effective in maintaining hepatocyte viability and liver-specific metabolic function as compared to that of single hepatocytes. These study findings suggested that ECM and cell-to-cell could recover cryopreserved hepatocytes from freezing/thawing injury to some extent. However, it is rarely reported in current literature how to improve culture of cryopreserved hepatocytes after thawing. Moshage *et al.* (1988) used precoating with homologous ECM to successfully reverse the deleterious effect of freezing/thawing on hepatocyte attachment efficiency and survival *in vitro* for over 4 weeks. The present work demonstrated for the first time that MSCs co-culture could also improve cellular activity and liver-specific metabolic function of cryopreserved hepatocytes as for fresh hepatocytes with a similar effect size. Gu *et al.* (2009[c]) observed extensive deposition of ECM, such as fibronectin, laminin, and collagen type I, III, and V, in MSCs co-culture system, while silencing of genes encoding fibronectin, laminin, and collagen type I and V in MSCs could reduce albumin secretion and urea synthesis of co-cultured hepatocytes. It is also likely that MSCs protect the host cells from oxidative stress by secreting more superoxide dismutase 3 in the presence of inflammatory cytokines, such as TNF-α and interferon γ (Kemp *et al.*, 2010).

3.4.2.4 Paracrine mechanisms of MSCs co-culture hepatotrophic effect

Hepatotrophic effect of MSCs co-culture is thought to result from three major aspects, including soluble cytokines secreted by MSCs, such as HGF, stem cell factor (Hu and Colletti, 2008), and other growth factors, ECM, and heterotypic cell-to-cell contact (Gómez-Aristizábal and Davies, 2012). A large number of previous studies have documented contribution of soluble factors to trophic effect of MSCs on co-cultured cells of epithelial origin, such as islet cells (Park *et al.*, 2010),

intestinal epithelial cells (Weil *et al.*, 2009), and hepatocytes (Murakami *et al.*, 2004), using the Transwell culture system. In this culture system, MSCs are separated from seed cells by the semipermeable culture insert to exclude the involvement of ECM and cell-to-cell contact but allow free transport of soluble cytokines and growth factors.

BM-MSCs CM exhibited a potent trophic effect on rat hepatocytes with respect to albumin secretion and urea synthesis as compared to hepatocyte or co-culture CM although CM culture did not improve spheroid hepatocyte formation (Ijima *et al.*, 2008). Gu *et al.* (2009[a]) further performed IL-6 neutralisation experiment and confirmed involvement of IL-6 in hepatotrophic effect of porcine BM-MSCs co-culture with respect to albumin secretion and urea synthesis. A recently published study regarding AT-MSCs co-culture with islet cells demonstrated that AT-MSCs CM improved porcine islet cell viability in a VEGF- rather than IL-6-dependent manner (Yamada *et al.*, 2013). Mintz *et al.* (2014) also recently reported that $CD34^+$ HSCs secreted 32 growth factors or cytokines associated with cell proliferation, survival, tissue repair, and wound healing. $CD34^+$ stem cells CM also prevented chemically-induced hepatocyte death by inhibiting the caspase-3 signalling pathway.

It remains controversial whether soluble factors released from MSCs also have a trophic effect on human hepatocytes. Fouraschen *et al.* (2012) reported that human liver-derived MSCs CM significantly upregulated expression levels of genes associated with protein synthesis, cell survival, and cell proliferation in human hepatocyte-like Huh7 cells. However, Fitzpatrick and her colleagues (Cell Transpl, Epub ahead of print) reported that neither MSCs indirect co-culture (MSCs separated from hepatocytes by a porous membrane) nor MSCs co-culture CM had minimal or insignficant trophic effect on human hepatocytes, in the absence of additional ECM deposition and hepatocyte-to-MSC contact as shown in the present work. Minimal hepatotrophic effect of MSCs indirect co-culture suggested that release of MSCs-derived trophic factors may require the extrinsic stimulation of heterotypic contact between MSCs and hepatocytes. Furthermore, insignficant hepatotrophic effect of MSCs co-culture CM implied a possibility that human hepatocytes did not respond well to MSCs-released trophic factors *in vitro*. Isolation of primary human hepatocytes is known to be technically more complex and time-consuming than that of animal-sourced hepatocytes. As a result, human hepatocytes may be in a relatively stressful status after isolation as compared to counterpart animal cells, and become less responsive to trophic stimuli. Taking significant hepatotrophic effect of MSCs direct co-culture, trophic effect of MSCs-derived soluble factors may require the synergistic effect of MSCs-derived ECM and hepatocyte-to-MSC interaction.

3.4.3 Antiapoptotic effect of MSCs co-culture on hepatocytes

Overall trophic effect of MSCs co-culture on liver-specific metabolic function of hepatocytes may result from two aspects, namely, increased hepatocyte survivability, potentiated metabolism of surviving hepatocytes, or both. It has been well documented in current literature that MSCs co-culture significantly improved hepatocyte survival (Gu *et al.*, 2009[a]; Gu *et al.*, 2009[b]; Gu *et al.*, 2009[c]); however, there exists a knowledge gap whether MSCs inhibit apoptosis and/or necrosis of co-cultured hepatocytes. Fitzpatrick and her colleagues (Cell Transpl, Epub ahead of print) showed

that co-culture with UC-MSCs suppressed caspase-mediated apoptosis of hepatocytes within one month of culture *in vitro*. The present work further confirmed that AT-MSCs significantly reduced both spontaneous apoptotic and necrotic death of co-cultured hepatocytes without switching the death mode.

Spontaneous apoptosis in mono-cultured hepatocytes is known to be a major cause of cellular loss of primary hepatocytes after culture *in vitro*. Spontaneous apoptotic death is controversially attributed to intracellular accumulation of nitric oxide following enzymatic digestion, which is invovled in the balance between proapoptotic and antiapoptotic effects on hepatocytes both *in vitro* and *in vivo* (Kim *et al.*, 2000). Cleavage of CK18 by caspases is an early event in cellular apoptosis. Increased serum CCK18 level has been observed in patients afflicted with liver cirrhosis, primary graft dysfunction, ALF (Hetz *et al.*, 2007), or non-alcoholic steatohepatitis (Yilmaz *et al.*, 2009), suggesting hepatocyte apoptosis and liver degeneration *in vivo*. Caspase signalling is reported to be invovled in rat hepatocyte apoptosis induced by endotoxin as mediated by TNF-α and downstream TNF-α receptor 1 in Kupffer cells (Hamada *et al*., 1999). Cleavage of CK18 at the position detected by the M30 CytoDeath™ ELISA kit used in the present work was reported to be initiated by caspase-9 and executed by caspases-3 and -7 (Schutte *et al.*, 2004), which could be inhibited by the caspase-inhibitor zVAD-fmk (Hägg *et al.*, 2002). The present work also demonstrated that MSCs specifically suppressed staurosporine-induced apoptosis rather than necrosis of co-cultured hepatocytes, while staurosporine treatment is known to activate caspase-3 signalling independently of caspases-8, -9, and -12 (Feng and Kaplowitz, 2002). Thus, reduction in CCK18 release from co-cultured hepatocytes as shown in the present work suggests that downregulation of caspase signalling may be implicated in antiapoptotic effect of MSCs co-culture on hepatocytes.

MSCs inhibit apoptosis of epithelial cells *via* paracrine mechanisms. Human BM-MSCs CM enriched with IL-6, HGF, and VEGF enhances the viability and proliferation of hypoxia-injured human foetal intestinal epithelial cells with concomitantly downregulated caspase-3 expression (Weil *et al.*, 2009). Rat islet cells co-cultured with BM-MSCs exhibited significantly upregulated expressions of antiapoptotic genes, such as *Mapkapk-2*, *Tnip-1*, and *Bcl-3*, concomitantly accompanied by increased IL-6 and TGF-β in the CM (Karaoz *et al.*, 2010). Park *et al.* (2010) reported human UC-MSCs CM containing high-level IL-6 and -8, VEGF, HGF, and TGF-β had a significant antiapoptotic effect on mouse islet cells both *in vitro* and *in vivo*. Yeung *et al.* (2012) showed that human BM-MSCs protected human islet cells from cellular apoptosis induced by interferon γ, TNF-α, and IL-1β; cytoprotective factors secreted by BM-MSCs included HGF and matrix MMP-2 and 9. Du *et al.* (2013) infused concentrated rat MSCs CM into rat liver graft, which resulted in reduction of hepatocyte apoptosis and concomitant increase of VEGF and MMP-9. Xagorari *et al.* (2013) also reported that BM-MSCs CM containing IL-6 protected hepatocytes from CCl_4-induced apoptosis through activation of fibroblast-like-protein signalling. However, the present work demonstrated paracrine factors contributed minimally to antiapoptotic effect of MSCs co-culture. This inconsistence might be due to the possibility that isolated human primary hepatocytes became unresponsive to antiapoptotic factors released from MSCs and present in MSCs CM.

3.4.4 Conclusions

In conclusion, the present work demonstrated that autologous liver NPCs had a significant trophic effect on human hepatocytes even at a very low co-culture density, although reproducibility and consistency of liver NPCs expansion *in vitro* remained debatable. Co-culture with AT-MSCs exhibited a significant trophic effect similar to those with BM- and UC-MSCs as compared to ADFs co-culture and hepatocyte mono-culture, while the effect size remained significant even at a low MSC:hepatocyte ratio. This suggests that hepatotrophic effect of MSCs co-culture derives from stem cell-specific factors, through a series of amplification effects. As optimisation of MSCs source and seeding density failed to further potentiate hepatotrophic effect of MSCs co-culture as shown in the present work, preconditioning of MSCs may be an effective alternative for optimising MSCs co-culture system *in vitro*.

Hepatotrophic effect of MSCs co-culture was also effective for steatotic and cryopreserved hepatocytes with a similar effect size for fresh, nonsteatotic hepatocytes. Moreover, MSCs co-culture significantly suppressed caspase-mediated apoptosis and necrosis of hepatocytes, and specifically inhibited staurosporine-induced hepatocyte apoptosis associated with caspase signalling activation. The absence of trophic and antiapoptotic effects on human hepatocytes as seen in MSCs indirect co-culture and co-culture CM culture also implied a possibility that isolated human primary hepatocytes became unresponsive to paracrine stimuli from MSCs. Therefore, ECM and heterotypic interaction between MSCs and hepatocytes synergizing paracrine mechanisms are more likely to significantly contribute to hepatotrophic and antiapoptotic effects of MSCs co-culture, underlying which MSCs co-culture may down- and upregulate apoptosis- and antiapoptosis-associated gene expressions of hepatocytes primarily by non-paracrine mechanisms.

CHAPTER 4 HYPOXIC PRECONDITIONING POTENTIATES HEPATOTROPHIC EFFECTS OF MSCS CO-CULTURE

4.1 Introduction

4.1.1 *Hepatotrophic effect of MSCs co-culture*

A number of previous studies demonstrated that MSCs co-culture improved survival, cellular activity, and liver-specific metabolism, such as albumin secretion, urea synthesis, and CYP450 activity, of hepatocytes. MSCs support co-cultured hepatocytes through soluble factors, ECM, and cell-cell crosstalk in a three-dimensional architecture, which synergistically mimics a physiological microenvironment for hepatocytes *in vitro*. The work in Chapter 2 further validated that MSCs co-culture had hepatotrophic effects and also applied to steatotic hepatocytes and cryopreserved hepatocytes, both of which are frequently encountered in HCT practice. Moreover, MSCs also inhibited spontaneous and chemically-induced apoptotic death of co-cultured hepatocytes, which might be a major contributive factor of MSCs.

In the previous chapter, effort was made to optimise MSCs/hepatocyte co-culture system by testing three types of MSCs, namely, AT-, BM-, and UC-MSCs at an empirical seeding ratio of 1:2.5 (MSC:hepatocyte). This finding suggested that even a very small number of MSCs were sufficient to maintain and improved co-cultured hepatocyte metabolism. There is a knowledge gap existing in current literature how to further potentiate MSCs hepatotrophic effect, which is expected to be technically less complex, biologically effective, and, more importantly, subject to no safety concern.

4.1.2 *Preconditioning of MSCs*

MSCs have been widely investigated for cellular replacement therapy and regenerative medicine. These multipotent cells can be driven to differentiate into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes in the presence of specific extrinsic and intrinsic cues (Khan and Hardingham, 2012). MSCs also promote repair and regeneration of the injured cells and tissues, such as fibrotic liver (Tsai *et al.*, 2009) and infarcted myocardium (Carvalho *et al.,* 2013), through paracrine and nonparacrine mechanisms. However, it remains undetermined whether MSCs can maintain their biological and physiological activities after consecutive expansion *in vitro* and transplantation *in vivo*. Human MSCs will become replicatively senescent and (epi)genetically unstable through passages and consequently have their proliferation and differentiation potential impaired. An increasing number of chemically-defined culture media have been tested on *in vitro* priming of MSCs to ensure reliable engraftment and long-term therapeutic effect after transplantation (Tonti and Mannello, 2008). MSCs are frequently preconditioned using pharmacological (Wisel *et al.*, 2009), biochemical and molecular (Khan *et al.*, 2011), and physical techniques (Rosová *et al.*, 2008) to optimise cellular functionality and improve cell graft survival, especially for transplantation to an inflammatory microenvironment (Herrmann *et al.*, 2010).

4.1.2.1 Pharmacological preconditioning

Lipopolysaccharide (LPS), the major component of the outer membrane of the Gram-negative bacteria, acts as an endotoxin and can elicit potent immune and inflammatory responses in humans and animals. Human BM-MSCs subjected to LPS secrete significantly higher levels of VEGF, FGF2, HGF, and IGF-1 *in vitro* by an NFκB- but not JNK-dependent mechanism (Crisostomo *et al.*, 2008). LPS-preconditioned MSCs exhibit an enhanced survival after engraftment into the myocardium by upregulating VEGF expression, which consequently improve *in vivo* neovascularisation and cardiac function (Yao *et al.*, 2009). LPS preconditioning can also protect MSCs from induced apoptosis. Wang *et al.* (2013) reported that low-dose LPS preconditioning preserved the mitochondrial transmembrane potential and inhibited Cyc-c release in rat BM-MSCs subjected to hypoxia and serum deprivation. Moreover, LPS preconditioning regulates MSCs immunomodulative effect. Mei *et al.* (2013) reported that LPS challenge significantly upregulated expression of inflammatory cytokines such as IL-1α, -1β, -6, and -8, and inhibitory immune mediators, such as indoleamine 2,3-dioxygenase 1, cyclooxygenase 2, interferon β, and MMP-2, but downregulation of MMP-9, in human UC matrix-derived MSCs, mediated by the toll-like receptor. However, the use of LPS-preconditioned MSCs for HCT is inevitably subject to the safety concern as endotoxin contamination is a major hazard to human subjects receiving cellular replacement therapy (Ra *et al.*, 2011).

4.1.2.2 Biochemical and molecular preconditioning

MSCs can be preconditioned with extrinsic cytokines and growth factors. MSCs exert the therapeutic effect by engrafting into the injured tissue possibly transdifferentiating into parenchymal cells and endothelial cells and/or secreting cytokines and growth factors. These soluble factors reciprocally act on MSCs to improve MSCs survival, mediate cellular functionality, and enhancing neoangiogenesis. Stromal-derived factor 1 (SDF-1), also known as C-X-C motif chemokine 12, is a chemokine protein that recruits immune and endothelial cells and regulates angiogenesis. SDF-1 preconditioning significantly suppresses apoptosis in rat BM-MSCs subjected to hypoxia and serum deprivation (Chen *et al.*, 2009). Preconditioning with diazoxide, a mitochondrial ATP-sensitive potassium channel agonist, can improve the survival rate of transplanted rat BM-MSCs by upregulating expression of bFGF and HGF, which reduces the myocardial infarction area and improves left ventricular function in rat myocardial infarction model (Cui *et al.*, 2010). TGF-α upregulates rodent BM-MSCs secretion of VEGF, which can be further potentiated by the synergistic effect of TNF-α; TGF-α-preconditioned MSCs show a better therapeutic effect on rat ischaemia/reperfusion myocardium as mediated by downregulation of IL-1β, TNF-α, and caspase-3 signalling pathways (Herrmann *et al.*, 2010). Moreover, MSCs isolated from a diseased donor may exhibit an impaired functionality due to the underlying biochemical insufficiency or genetic defect, which can be compensated or repaired by growth factor preconditioning. BM-MSCs isolated from streptozotocin-induced diabetic mice were preconditioned with IGF-1 and concomitant FGF-2; these preconditioned MSCs exhibited a significantly higher superoxide dismutase activity, less cellular

apoptosis, stronger *in vitro* tube-forming ability, and improved chemotactic mobility (Khan *et al.*, 2011).

More importantly, preconditioning with soluble molecules was reported to potentiate the trophic and resuscitation capacity of MSCs; human AT-MSCs preconditioned with a combination of hyaluronic, butyric, and retinoic acids optimised islet cell graft revascularisation after intrahepatic co-transplantation, as mediated by VEGF, kinase insert domain receptor, and HGF signalling pathways, and improved glycaemic control in diabetic rats (Cavallari *et al.*, 2012). However, preconditioning with cytokines and growth factors, which has been validated to be effective on the bench, is not feasible and cost-effective for the purpose of bedside transplantation requiring a large number of MSCs to be preconditioned. Additionally it remains unknown whether preconditioning effect of these cytokines and growth factors can maintain in the long term after transplantation *in vivo*. Genetic engineering targeting at these favourable molecules, such as VEGF (Shevchenko *et al.*, 2013), HGF (Yu *et al.*, 2007; Wang *et al*., 2013), and TGF-β (Guo *et al.*, 2006; Xue *et al.*, 2013), may be a solution to this uncertainty, while gene modification itself is technically complex and subject to some safety risks.

4.1.2.3 Hypoxic preconditioning

MSCs normally reside in a physiologically hypoxic niche, such as AT, BM, and UC. Hypoxia plays an important role in the fine equilibrium between proliferative and differentiation potentials of MSCs as mediated by a number of extrinsic and intrinsic signals. A long-term low-oxygen tension culture environment maintains MSCs in an undifferentiated, multipotent status. Human BM-MSCs cultured under 1%-O_2 hypoxia showed a significantly increased proliferative ability and migration capability, underlying which were upregulated expression of stemness genes, such as *OCT4*, *NANOG*, *SALL4*, and *KLF4* (Weijers *et al.*, 2011; Hung *et al.*, 2012). Hypoxic culture condition also determines the differentiation fate of MSCs towards osteoblasts, chondrocytes, and adipocytes. Hypoxia does not alter the immunophenotype or compromise the multi-lineage differentiation potential of BM-MSCs; however, hypoxia inhibits the transdifferentiation of MSCs into osteoblasts signalled by MAPK/ERK 1/2 (Wang *et al.*, 2012), hypoxia-induced factor-1α (HIF-1α; Hsu *et al.*, 2013), and *Notch1* (Xu *et al.*, 2013). In contrast, MSCs exposed to chondrogenic growth factors and a 2% O_2 hypoxic environment express significantly greater collagen type II and proteoglygan, two major components of cartilage (Kanichai *et al.*, J Cell Physiol, 2008). Moreover, hypoxia mobilises MSCs into the circulating bloodstream in a way similar to HSCs (Rochefort *et al.*, 2006), and promotes the domiciliation of MSCs to the injured site (Rochefort *et al.*, 2005).

Hypoxic preconditioning (HPc), namely, *in vitro* hypoxic culture followed by normoxic culture or transplantation *in vivo*, has been widely applied to optimise MSCs for uses of regenerative medicine and tissue engineering. HPc can protect MSCs from hypoxia/reoxygenation-induced apoptosis by stabilising mitochondrial membrane potential, upregulating Bcl-2 and VEGF signalling, and promoting phosphorylation of ERK and Akt (Wang *et al.*, 2008). HPc significantly increases expression of pro-survival and pro-angiogenic factors, such as HIF-1α; angiopoietin 1, VEGF and

VEGF receptor, erythropoietin, Bcl-2, and Bcl-xL, and also significantly decreases that of caspase-3 initiating cellular apoptosis in mouse BM-MSCs; HPc-MSCs improve angiogenesis in myocardium and enhance morphological and functional recovery (Hu *et al.*, 2008). This potentiated therapeutic effect of transplanting HPc-MSCs has also been validated in experimental limb, cerebral, renal, and spinal cord ischaemia. HPc can rejuvenilise aged AT-MSCs by upregulating gene expression of pro-angiogenic factors, including VEGF, placental growth factor, and HGF but downregulating that of TGF-β (Efimenko *et al.*, 2011). Due to its technical simplicity and superior cost-effectiveness, HPc is a promising physical technique for optimising MSCs for cellular replacement therapy. However, it is rarely reported in literature whether HPc can potentiate the trophic and protective effects of MSCs on co-cultured or co-transplanted seed cells.

4.1.3 *ROS: a signalling factor pivoting HPc*

ROS refer to a collection of chemically reactive, oxygen-containing molecules, including radical, superoxide anion, hydrogen peroxide, and singlet oxygen. Production of ROS can occur in both physiological and pathological conditions, mainly in response to oxidative stress but also to exogenous sources, such as ionizing radiation. As a natural by-product of normal oxygen metabolism, excessive ROS may cause damage to cells by disrupting DNA and oxidising fatty acids, amino acids, and specific enzymes. However, ROS plays a crucial role in regulating cellular signalling and maintaining homeostasis. ROS are controversially thought to be involved in ageing, and also extensively studied in the scenario of cancer, including carcinogenesis, uncontrolled proliferation, escape from apoptosis, tumor cell invasion, neoangiogenesis and metastasis, and chronic inflammation bridging cancer.

Exogenous ROS induce marked apoptosis of MSCs in a dose- and time-dependent manner through the endoplasmic reticulum and mitochondrial pathways, including p38 MAPK signalling at an early time point and c-Jun N-terminal kinase signalling at a late time point (Wei *et al.*, 2010). MSCs, however, are well known to be resistant to the detrimental effect of excessive ROS by possession of a potent ROS scavenging facility (Valle-Prieto and Conget, 2010). Conversely HPc is known to significantly increase intracellular ROS activity and improve MSCs survival and motility. ROS plays a crucial role in maintaining undifferentiated status of human MSCs: decreased intracellular ROS activity accompanies transdifferentiation of MSCs into osteoblasts, while exogenous hydrogen peroxide prevents osteogenic differentiation (Chen *et al.*, 2008). In contrast, increased intracellular ROS from upregulated expression of NAD(P)H oxidase 4 facilitates adipocyte differentiation of mouse embryonic MSCs by activating the transcription of cAMP response element-binding protein (Kanda *et al.*, 2011). ROS controls the cell growth cycle and life span of human MSCs *in vitro* as mediated by upregulation of p38 MAPK signalling. ROS is also reported to regulate migration and tube formation (*in vitro* angiogenesis) of MSCs as mediated by placental growth factor signalling (Shyu *et al.*, 2008). Increase of intracellular ROS results in significant downregulation of focal adhesion-related molecules, such as phospho-FAK (focal adhesion kinase) and phospho-Src, and integrin-related adhesion molecules, such as integrin 5α and

β1, suggesting the involvement of ROS in MSCs mobilisation and migration (Song *et al.*, 2010). It is possible that intracellular ROS might mediate the hypothesised HPc-induced potentiation of MSCs hepatotrophic and antiapoptotic effects on co-cultured hepatocytes.

4.1.4 *Chapter objectives*

- *Effects of HPc on MSCs*

To investigate the effects of HPc on AT-MSCs *in vitro*, with respect to cellular mitochondrial dehydrogenase activity, attachment, DNA/protein synthesis, and intracellular ROS activity.

- *Optimisation of time length of HPc*

To compare potentiative effect of 8-, 24-, 48-, and 72-h HPc on MSCs co-culture hepatotrophic effect, with respect to cellular viability, cell attachment, and liver-specific metabolism.

- *Potentiation of MSCs co-culture paracrine mechanisms by HPc*

To investigate whether HPc potentiates MSCs co-culture hepatotrophic effect by enhancing paracrine mechanisms.

- *Potentiation of MSCs co-culture antiapoptotic effect*

To investigate whether MSCs can protect co-cultured hepatocytes from chemically-induced cell apoptosis.

- *Dependence of HPc-induced potentiative effects on intra-MSCs ROS activity*

To investigate whether HPc induces potentiation of MSCs co-culture hepatotrophic and antiapoptotic effects in an intra-MSCs ROS activity dependent manner.

4.2 Materials and methods

4.2.1 *Subculture and immunophenotyping of AT-MSCs*

Human AT-MSCs were subcultured as described in ***Section 2.2*, *Subculture of MSCs***. P6–8 MSCs were characterised using flow cytometry with a human MSC phenotyping kit (Miltenyi Biotec Ltd., Surrey, UK). These MSCs were stained positive for CD73, CD90, and CD105, and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface antigens (Dominici *et al.*, 2006).

4.2.2 *Isolation of human hepatocytes*

Human donor liver tissues were processed as described in ***Section 2.3*, *Primary Harvest of Human Hepatocytes***. The total number and viability of fresh hepatocytes were determined using a hematocytometer and the trypan blue exclusion technique with a light microscope. Red blood cells in hepatocyte pellet were lysed using sterile water for injection for 1 min at room temperature, followed by centrifugation. The batch of hepatocytes with a viability of over 60% on trypan blue exclusion was used for further experiments.

4.2.3 *Optimisation of HPc*

P6–8 MSCs were subjected to hypoxia (HPc-MSCs) using an air-tight and moistened hypoxia incubator chamber (StemCell Technologies SARL, Sirocco, France; Figure 4.1), at 37°C for 8 h, 24 h, 48 h, and 72 h, respectively. The chamber was purged with a mixture of 95% N_2/5% CO_2 (BOC Special Gases, Surrey, UK) for 3 min, and the flow rate was set at 20 L/min using a single flow meter (Billups-Rothenberg Inc., Del Mar, CA, USA) as recommended by the manufacturer. The intra-chamber oxygen saturation measured 2% (hypoxia) using a GasBadge® Plus single-gas monitor (Industrial Scientific, Arras, France) upon the completion of purge. MSCs that were cultured at an atmosphere of 95% air/5% CO_2 served as control (NPc-MSCs), and the intra-incubator oxygen saturation measured 20% (normoxia) using the same protocol. MSCs morphology was examined using a standard light microscope. The experiments were performed in duplicate and repeated in triplicate independently. The optimal time length of HPc that exhibited the greatest potentiative effect on MSCs co-culture hepatotrophic effect was used in further experiments.

4.2.4 *Hepatocyte co-culture protocols*

4.2.4.1 Direct co-culture of hepatocytes with MSCs

Fresh hepatocytes were co-cultured with HPc- *versus* NPc-MSCs as described in ***Section 2.4*, *Hepatocyte Mono-culture and Co-culture***. The seeding density of MSCs was 20,000 viable cells per cm^2, and that of hepatocytes was 50,000 viable cells per cm^2, at a MSC:hepatocyte ratio of 1:2.5.

Figure 4.1 Hypoxia incubator chamber (left panel) and single flow meter (right panel). Air-tight and moistened hypoxia incubator chamber was purged with a mixture of 95% N_2/5% CO_2 at a flow rate of 20 L/min to produce a hypoxic environment at an intra-incubator oxygen saturation measuring 20% (adapted from www.stemcell.com).

Hepatocytes co-cultured with NPc-MSCs were used as control, and mono-cultured HPc- and NPc-MSCs were used as blank controls.

4.2.4.2 Indirect co-culture of hepatocytes with MSCs

Indirect co-culture using Transwell Hepatocytes were indirectly co-cultured with HPc- *versus* NPc-MSCs using Transwell® Permeable Supports, as described in ***Section 3.2.5.5***, ***Indirect co-culture of hepatocytes with MSCs***, to investigate whether HPc could potentiate contribution of MSCs-derived soluble factors to MSCs co-culture hepatotrophic effect.

MSCs co-culture CM AT-MSCs were plated onto T75 culture flasks at a density of 20,000 viable cells per cm^2, and fed with hepatocyte culture media for 24-hour pre-culture at 37°C. Mono-cultured MSCs were subjected to 2%-O_2 HPc and 20%-O_2 NPc for 24 h, respectively. Fresh hepatocytes were cultured with HPc- *versus* NPc-MSCs co-culture CM as described in ***Section 3.2.5.5*, *Indirect co-culture of hepatocytes with MSCs***, to investigate whether HPc could potentiate paracrine contribution of MSCs co-culture to hepatotrophic effect.

The culture media were refreshed on days 1, 3, 5, and 7, respectively. The cell morphology was examined using a standard inverted light microscope equipped with a digital SLR camera. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for further experiments. Cell cultures were rinsed with one-wash PBS at room temperature for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.5 *Characterisation of HPc effects on MSCs*

4.2.5.1 General cellular activity

P6–8 AT-MSCs at 80% confluency were subjected to 2%-O_2 hypoxia or 20%-O_2 normoxia for 24 h. MTT assay was performed as described in ***Section 2.5*, *3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium Bromide Colorimetric Assay*** to determine MSCs mitochondrial dehydrogenase

activity. SRB assay was performed as described in ***Section 2.6, Sulforhodamine B Colorimetric Assay*** to determine overall MSCs attachment. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.5.2 3H-thymidine and 14C-leucine incorporation assays

^{3}H-thymidine and ^{14}C-leucine incorporation assays were used to examine effects of HPc *versus* NPc on MSCs DNA and protein syntheses. P6–8 MSCs at 80% confluency were fed with MSCs culture media containing ^{3}H-labled thymidine and ^{14}C-labled leucine (Amersham International, Amersham, UK), at a concentration of 20 μCi/mL and 1 μCi/mL, respectively. These MSCs were further subjected to 24-hour hypoxia or normoxia. Upon the completion of preconditioning, radioisotope-labelled cells were harvested onto glass fibre membranes using a Packard FilterMate (Packard Instrument Co., Ltd., Caversham, UK), and the filters were dried at 60°C for 1 h. The radioactivities were counted using a Packard Matrix 9600 β-counter (Packard Instrument Co., Ltd., Caversham, UK), and expressed as count per min per well (cpm/well). MSCs incubated in radioisotope-free culture media were used as blank control. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.5.3 Quantitation of intra-MSCs ROS activity

Intracellular ROS activity was measured using flow cytometry with dichloro-dihydrofluorescein diacetate acetyl ester (DCFDA) staining as previously described by Eruslanov and Kusmartsev (2010). DCFDA, a cell-permeant fluorogenic dye, is deacetylated by cellular esterases to non-fluorescent 2',7'-dichlorofluorescin (DCFDH), which is subsequently oxidised by intracellular hydroxyl, peroxyl and other forms of ROS into cell-impermeant 2'-7'-dichlorofluorescein (DCF). DCF can be detected by fluorescence spectroscopy at the maximum excitation and emission spectra of 495 nm and 527 nm, respectively.

Briefly, P6–8 AT-MSCs were plated onto non-tissue-culture-treated 6-well plates. On 80% confluency, MSCs were subjected to 24-hour HPc *versus* NPc and rinsed with one wash of PBS, 1 mL per well. Cell cultures were incubated with PBS, 0.5 mL per well, containing 2.5-μM 5,6-chloromethyl-2',7'-DCFDA (Molecular Probes, Inc., Eugene, OR, USA) specifically designated for liver cell staining, at 37°C for 30 min. HPc-MSCs incubated with plain PBS were used as blank control, and NPc-MSCs deprived of serum 24 h prior to labelling were used as positive control. Cells were rinsed with one wash of PBS, 1 mL per well, and detached from the plates using EDTA-chelated trypsin (TrypLE™ Select; Invitrogen Ltd, Paisley, UK), 0.5 mL per well. Dissociation was terminated by adding PBS containing 10% FCS, 1 mL per well. Cell suspension was centrifuged at 4°C and 1,500 rpm for 5 min. Cell pellets were resuspended in 0.5-mL plain PBS at a density of 100,000 cells per mL, and kept on ice and in the dark for further flow cytometry.

An 8-channel BD FACS Canto II flow meter (BD Biosciences, San Jose, CA, USA) was used to quantitate intra-MSCs ROS activity. Briefly, 10,000 events were recorded and analysed, and the side scatter (SSC) axis was plotted against the forward scatter (FSC) axis to produce the scatter

graph and gate the cell population (at least 90% of the total events). Unlabelled cells were analysed to polygon gate the positive population (labelled cells). Intracellular ROS activity was measured using the Alex Fluor® 488 fluorescein isothiocyanate (FITC) channel, and the SSC-axis was plotted against the FITC-axis. The flow cytometry analysis software FlowJo 10.0.6 for Windows (Tree Star, Inc., Ashland, OR, USA) was used to determine median fluorescence intensity (FI) for each sample. Median FIs of HPc-, and serum-deprived NPc-MSCs were normalised to those of NPc-MSCs (Du *et al.*, J Biol Chem, 2006). All experiments were performed in duplicate and repeated in triplicate independently.

4.2.6 *NAC antagonisation experiment*

4.2.6.1 Optimisation of NAC concentration

P6–8 AT-MSCs were plated onto non-tissue culture-treated 6-well plates. On 80% confluency, MSCs were and rinsed with one wash of PBS, and subjected to 24-hour HPc *versus* NPc and rinsed with one wash of PBS, 1 mL per well. HPc-MSCs were pretreated with 0-, 5-, 10-, and 20-mM NAC (PLIVA Pharma, Ltd., Hampshire, UK), respectively. Intra-MSCs ROS activity was measured as described in *Section 4.2.5.3, Quantitation of intra-MSCs ROS activity*, and the least concentration of NAC that resulted in significantly lower ROS activity in HPc-MSCs than that in NPc-MSCs was used for further antagonisation experiments. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.6.2 Co-culture of hepatocytes with non-pretreated and NAC-pretreated HPc-MSCs

P6–8 AT-MSCs were plated onto collagen-coated 24-well plates and subjected to 24-hour HPc; HPc-MSCs were pretreated with the optimal concentration of NAC and without NAC, respectively. MSCs cultures were rinsed with one wash of PBS, and fresh hepatocytes were co-cultured with HPc-MSCs as described in *Section 4.2.4.1, Direct co-culture of hepatocytes with MSC*. The culture media were refreshed on days 1, 3, 5, and 7, respectively. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for further albumin, urea, CCK18, and CK18 assays. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.7 *General cellular activity and liver-specific metabolic function assays*

MTT assay was performed as described in ***Section 2.5*, *3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium Bromide Colorimetric Assay*** to determine hepatocyte overall viability. SRB assay was performed as described in ***Section 2.6*, *Sulforhodamine B Colorimetric Assay*** to determine overall hepatocyte attachment. Albumin ELISA was performed as described in ***Section 2.7*, *Albumin Enzyme-linked Immunoabsorbent Assay*** to determine protein synthesis of hepatocytes. Urea colorimetric assay was performed as described in ***Section 2.8*, *Urea Colorimetry*** to determine nitrogen detoxification of hepatocytes. All experiments were performed in duplicate and repeated in

triplicate independently.

4.2.8 *Staurosporine cytotoxicity assay*

Fresh hepatocytes were pre-treated with 1-μM staurosporine (Sigma-Aldrich, St. Louis, MO, USA) and co-cultured with non-NAC- and NAC-pretreated HPc-MSCs for 24 h, as controlled by staurosporine-treated hepatocytes co-cultured with NPc-MSCs. Cell culture supernatants were collected for CCK18 and CK18 assays as described in ***Section 2.9*****,** ***Caspase-cleaved CK18 Assay*** and ***Section 2.10*****,** ***CK18 Assay***. All experiments were performed in duplicate and repeated in triplicate independently.

4.2.9 *Hepatocyte apoptosis and total death assays*

CCK18 assay was performed as described in ***Section 2.9*****,** ***Caspase-cleaved CK18*** **Assay** to determine caspase-mediated hepatocyte apoptosis. CK18 assay was performed as described in ***Section 2.10*****,** ***CK18*** **Assay** to determine total hepatocyte death. The ratio of CCK18 to CK18 was also produced to determine the death mode of hepatocytes *in vitro*; a ratio of over 0.40 indicated that hepatocytes underwent apoptosis mainly, and necrosis *vice versa*. All experiments were performed in duplicate and repeated in triplicate independently.

4.3 Results

4.3.1 *Morphology of HPc- vs. NPc- MSCs mono- and co-cultures*

4.3.1.1 Morphology of HPc-MSCs

HPc did not result in cell detachment, necrosis, or morphological distortion of MSCs. HPc-MSCs (Fig. 4.2A) exhibited a spindle-shaped fibroblast-like phenotype similar to NPc-MSCs (Fig. 4.2B) and reached 80% confluency within 7 d. On confluency HPc-MSCs were also polarised, with a small cell body and multiple slim projections in a homogenous manner. HPc-MSCs showed viability over 99% on trypan blue exclusion after trypsinisation.

4.3.1.2 Morphology of HPc-MSCs co-culture

The great majority of freshly isolated human hepatocytes attached to the MSCs monolayer within 2–4 hours, and only a few hepatocytes were detached from culture vessel surface at the time of culture media replacement Hepatocytes co-cultured with HPc-MSCs exhibited a morphology similar to those in co-culture with NPc-MSCs (Figure 4.2C), but a little more hepatocytes aggregated into larger, oval-shaped colonies and attached close to HPc-MSCs over 7 days of co-culture (Figure 4.2D).

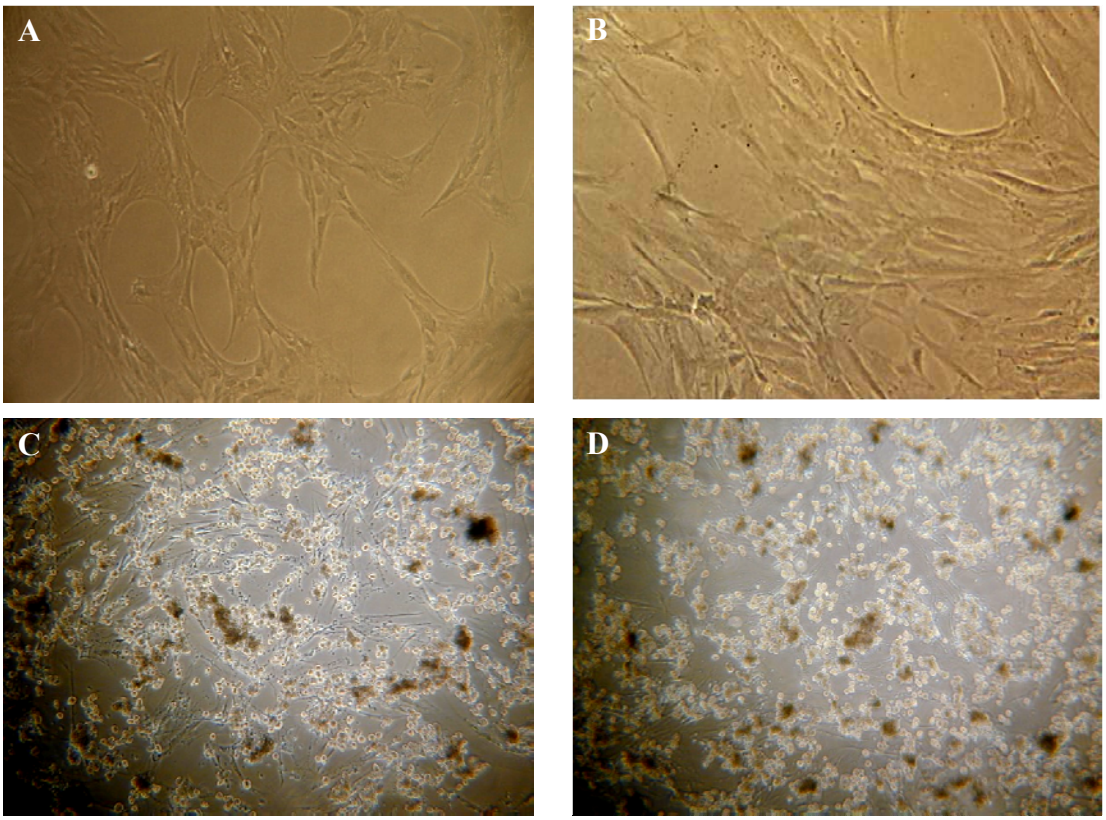

Figure 4.2 Morphology of NPc-MSCs (A), HPc-MSCs (B), and hepatocytes co-cultured with NPc- (C) and HPc-MSCs (D) (200×): HPc-MSCs (A) exhibited a spindle-shaped fibroblast-like phenotype similar to NPc-MSCs (B); compared to hepatocytes co-cultured with NPc-MSCs (C), a little more hepatocytes aggregated into larger, oval-shaped colonies in co-culture with HPc-MSCs (D).

4.3.2 *Effects of HPc on MSCs*

4.3.2.1 HPc has no cytotoxic effect on MSCs

As is shown in Figure 4.3A, HPc-MSCs showed a significant decrease in mitochondrial dehydrogenase activity as compared to NPc-MSCs (HPc *vs.* NPc, 1.18 ± 0.11 *vs.* 1.36 ± 0.09 OD units; P <0.05). However, HPc resulted in a significant increase in SRB cell attachment as compared to NPc (1.88 ± 0.11 *vs.* 1.66 ± 0.11 OD units; P <0.01). Addition of 10-mM NAC significantly reduced MTT activity (HPc+NAC, 0.95 ± 0.09 OD units; P <0.01 *vs.* HPc) and reversed cellular attachment increase (1.53 ± 0.14 OD units; P <0.01) in HPc-MSCs.

4.3.2.2 HPc increases MSCs DNA synthesis

As is shown in Figure 4.3B, HPc significantly increased DNA synthesis in MSCs as compared to NPc (3,121 ± 295 *vs.* 1,815 ± 334 cpm/well; P <0.01). However, HPc resulted in a significant reduction in MSCs protein synthesis as compared to NPc (567 ± 60 *vs.* 715 ± 118 cpm/well; P < 0.05). Addition of 10-mM NAC significantly reversed DNA synthesis increase (1,314 ± 54 cpm/well; P <0.01) and further reduced protein synthesis (263 ± 17 cpm/well; P < 0.01) in HPc-MSCs.

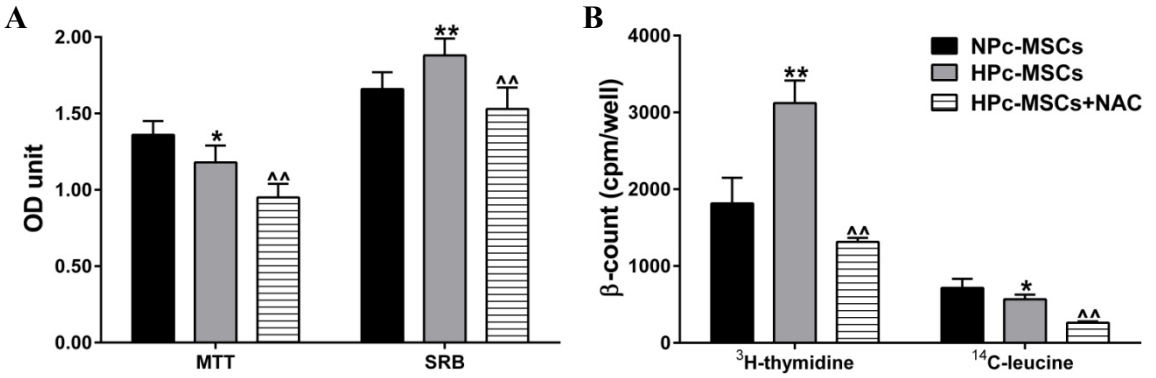

Figure 4.3 Effects of HPc and NAC pretreatment on MTT and SRB activities (A) and ^{3}H-thymidine and ^{14}C-leucine incorporation (B) of MSCs. HPc significantly decreased MSCs mitochondrial dehydrogenase activity but significantly increased cellular attachment, while NAC pretreatment significantly reduced MTT and SRB activities in HPc-MSCs (A); HPc also significantly increased DNA synthesis but significantly decreased protein synthesis, while NAC pretreatment significantly reduced DNA and protein syntheses in HPc-MSCs (B). All data were expressed as mean ± SD (error bar); *P <0.05 and **P <0.01 *versus* NPc-MSCs; ^^P <0.01 *versus* HPc-MSCs (n = 6). NPc, normoxia-preconditioned; HPc, hypoxia-preconditioned; NAC, N-acetylcysteine.

4.3.3 *Potentiative effect of HPc on MSCs co-culture hepatotrophic effect*

4.3.3.1 HPc potentiates MSCs co-culture hepatotrophic effect

Mitochondrial dehydrogenase activity HPc at all time lengths exhibited a limited potentiative effect on MSCs hepatotrophic effect, with respect to hepatocyte mitochondrial dehydrogenase activity, as compared to control NPc (Figure 4.4A). HPc co-culture did not significantly improve hepatocyte MTT activity on days 1 and 5; however, hepatocytes co-cultured

with HPc(8h)-, HPc(48h)-, and HPc(72h)-MSCs had a significantly higher MTT activity than those co-cultured with control NPc-MSCs on day 3 (NPc *vs.* HPc(8h) *vs.* HPc(24h) *vs.* HPc(48h) *vs.* HPc(72h), 1.45 ± 0.12 *vs.* 1.69 ± 0.17 *vs.* 1.62 ± 0.20 *vs.* 1.77 ± 0.14 *vs.* 1.65 ± 0.17 OD units; P <0.01), and hepatocytes co-cultured with HPc(24h)-MSCs had a significantly higher MTT activity than control NPc co-culture on day 7 (2.60 ± 0.25 *vs.* 2.75 ± 0.25 *vs.* 3.09 ± 0.33 *vs.* 2.79 ± 0.33 *vs.* 2.92 ± 0.30 OD units; P <0.05).

Cell attachment HPc at all time lengths exhibited a limited potentiative effect on MSCs hepatotrophic effect, with respect to hepatocyte cellular attachment, as compared to control NPc (Figure 4.4B). HPc-MSCs co-culture did not significantly improve hepatocyte SRB activity on days 1, 3, and 5; however, hepatocytes co-cultured with HPc(24h)- and HPc(48h)-MSCs had a significantly higher SRB activity than those co-cultured with control NPc-MSCs on day 7 (3.58 ± 0.42 *vs.* 3.93 ± 0.27 *vs.* 4.14 ± 0.29 *vs.* 4.26 ± 0.29 *vs.* 4.03 ± 0.28 OD units; P <0.05).

Albumin secretion No albumin secretion was detected in NPc- and HPc-MSCs mono-culture. HPc at 24, 48, and 72 h significantly potentiated MSCs hepatotrophic effect, with respect to albumin secretion, as compared to control NPc, although 8-h HPc did not show any potentiative effect throughout 7 days of co-culture (Figure 4.4C). HPc co-culture did not significantly improve hepatocyte secretion of albumin on day 1; however, hepatocytes co-cultured with HPc(24h)-, HPc(48h)-, and HPc(72h)-MSCs had a significantly higher albumin secretion, to a similar extent, than those co-cultured with control NPc-MSCs from day 3 (2.4 ± 0.3 *vs.* 2.4 ± 0.3 *vs.* 3.7 ± 0.4 *vs.* 3.6 ± 0.4 *vs.* 3.9 ± 0.4 μg/10^6 hepatocytes; P <0.01) until day 7 (4.6 ± 0.4 *vs.* 4.3 ± 0.3 *vs.* 5.4 ± 0.3 *vs.* 5.2 ± 0.3 *vs.* 5.4 ± 0.3 μg/10^6 hepatocytes; P <0.01).

Urea synthesis HPc at 24, 48, and 72 h significantly potentiated MSCs hepatotrophic effect, with respect to urea synthesis, as compared to control NPc, although 8-h HPc did not show any potentiative effect throughout 7 days of co-culture (Figure 4.4D). HPc co-culture did not significantly improve hepatocyte synthesis of urea on day 1; however, hepatocytes co-cultured with HPc(24h)-, HPc(48h)-, and HPc(72h)-MSCs had a significantly higher urea synthesis, to a similar extent, than those co-cultured with control NPc-MSCs from day 3 (23.4 ± 2.2 *vs.* 23.3 ± 2.3 *vs.* 32.8 ± 3.1 *vs.* 32.4 ± 2.4 *vs.* 33.2 ± 3.1 μg/10^6 hepatocytes; P <0.01) until day 7 (35.9 ± 3.0 *vs.* 33.5 ± 3.1 *vs.* 40.8 ± 3.6 *vs.* 40.4 ± 3.3 *vs.* 41.4 ± 3.5 μg/10^6 hepatocytes; P <0.01).

Overall 24-, 48-, and 72-h rather than 8-h HPc significantly potentiated MSCs co-culture hepatotrophic effect, to a similar extent, as compared to control 24-h NPc. Therefore, 24 h was determined to be the optimal time length of HPc used in further hepatocyte co-culture experiments.

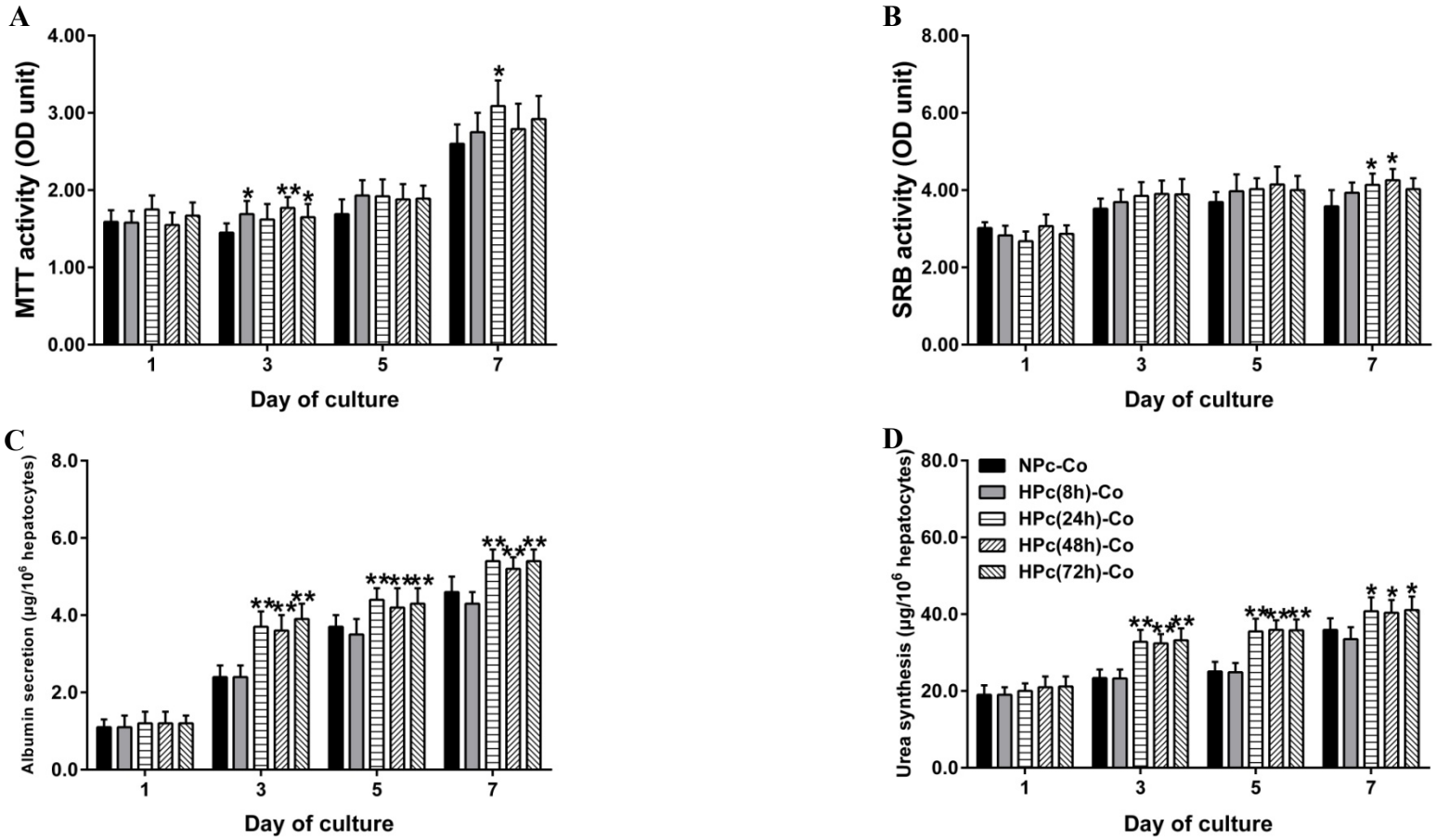

Figure 4.4 MTT activity (A), SRB cell attachment (B), albumin secretion (C), and urea synthesis (D) of hepatocytes co-cultured with HPc(8h)-, HPc(24h)-, HPc(48h)-, and HPc(72h)- MSCs *versus* hepatocytes co-cultured with NPc(24h)-MSCs at a MSC:hepatocyte seeding ratio of 2.5:1. HPc for 24, 48, and 72 h significantly potentiated MSCs co-culture hepatotrophic effect from day 3 until day 7. All data were expressed as mean ± SD; **P* <0.05 and ***P* <0.01 *versus* control NPc co-culture (*n* = 6). NPc, normoxia-preconditioned; HPc, hypoxia-preconditioned; Co, co-culture.

4.3.3.2 HPc does not potentiate MSCs paracrine contribution to co-culture hepatotrophic effect

HPc-MSCs indirect co-culture using Transwell plates exhibited no significantly potentiated hepatotrophic effect, with respect to hepatocyte mitochondrial dehydrogenase activity and cellular attachment (Table 4.1), as well as albumin secretion and urea synthesis (Table 4.2), as compared to control NPc-MSCs indirect co-culture, throughout 7 days of culture.

Table 4.1 MTT activity and SRB cell attachment (mean ± SD) of hepatocytes indirectly co-cultured on Transwell plates with HPc-MSCs *versus* with NPc-MSCs

	MTT (OD unit)				SRB (OD unit)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
HPc-iCo	1.58 ± 0.18	1.43 ± 0.21	1.35 ± 0.25	1.44 ± 0.20	3.03 ± 0.22	3.21 ± 0.24	3.10 ± 0.19	3.18 ± 0.25
NPc-iCo	1.60 ± 0.16	1.30 ± 0.10	1.25 ± 0.10	1.31± 0.09	2.88 ± 0.20	3.10 ± 0.20	3.01 ± 0.16	3.05 ± 0.21

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; iCo-, indirect co-culture.

Table 4.2 Albumin secretion and urea synthesis (mean ± SD) of hepatocytes indirectly co-cultured on Transwell plates with HPc-MSCs *versus* with NPc-MSCs

	Albumin (μg/10^6 hepatocytes)				Urea (μg/10^6 hepatocytes)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
HPc-iCo	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	18.6 ± 2.4	16.9 ± 2.5	17.1 ± 2.4	15.6 ± 2.0
NPc-iCo	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	18.6 ± 2.4	16.9 ± 2.5	17.1 ± 2.4	15.6 ± 2.0

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; iCo-, indirect co-culture.

HPc-MSCs co-culture CM improved hepatocyte mitochondrial dehydrogenase activity on day 3 only (HPc *vs.* NPc, 2.11 ± 0.25 *vs.* 1.21 ± 0.19 OD units; $P < 0.01$), but did not significantly increase hepatocyte attachment throughout 7 days of culture as compared to control NPc-MSCs co-culture CM (Table 4.3). HPc-MSCs co-culture CM also significantly increased hepatocyte secretion of albumin only day 7 only (1.4 ± 0.1 *vs.* 1.2 ± 0.1 μg/10^6 hepatocytes; $P < 0.01$), but did not significantly improve urea synthesis throughout 7 days of culture as compared to control NPc-MSCs co-culture CM (Table 4.4).

Table 4.3 MTT activity and SRB cell attachment (mean ± SD) of hepatocytes cultured with HPc-MSCs co-culture CM *versus* with NPc-MSCs co-culture CM

	MTT (OD unit)				SRB (OD unit)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
HPc-	1.54 ± 0.17	2.11 ± 0.25**	1.42 ± 0.23	1.38 ± 0.19	2.63 ± 0.19	2.90 ± 0.24	2.99 ± 0.20	2.98 ± 0.23
NPc-	1.58 ± 0.15	1.21 ± 0.19	1.25 ± 0.10	1.29 ± 0.17	2.71 ± 0.26	2.91 ± 0.24	2.93 ± 0.26	2.97 ± 0.24

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; **$P < 0.01$ *versus* NPc.

Table 4.4 Albumin secretion and urea synthesis (mean ± SD) of hepatocytes cultured with HPc-MSCs co-culture CM *versus* with NPc-MSCs co-culture CM

	Albumin (μg/10^6 hepatocytes)				Urea (μg/10^6 hepatocytes)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
HPc-	1.1 ± 0.1	1.1 ± 0.3	1.1 ± 0.2	1.4 ± 0.1**	18.0 ± 3.6	17.6 ± 2.6	17.2 ± 2.2	17.0 ± 2.2
NPc-	1.0 ± 0.1	1.0 ± 0.3	1.1 ± 0.2	1.2 ± 0.1	19.0 ± 2.6	15.6 ± 2.6	16.4 ± 2.3	15.6 ± 2.6

HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned**;** ***P*< 0.01 *versus* NPc.

4.3.4 *Potentiative effect of HPc depends on intra-MSCs ROS activity*

4.3.4.1 NAC pretreatment antagonises HPc-induced intra-MSCs ROS activity increase

As is shown in Figure 4.5, serum deprivation and HPc significantly increased intra-MSCs ROS activity to a similar extent as compared to NPc (normalised median FI: NPc *vs.* NPc+SF *vs.* HPc, 100.0% *vs.* 133.8% ± 16.4% *vs.* 141.1% ± 17.1%, P <0.01). Pretreatment with 5-mM NAC did not completely eliminate HPc-induced increase in ROS activity (HPc+NAC, 113.1% ± 11.2%, P <0.05 *vs.* NPc); however, addition of 10- (74.3% ± 7.2%, P <0.01) and 20-mM NAC (63.1% ± 3.9%, P <0.01) significantly antagonised HPc-induced ROS activity increase in HPc-MSCs. Therefore, 10-mM NAC was used in further antagonisation experiments.

4.3.4.2 HPc-potentiated co-culture hepatotrophic effect depends on intra-MSCs ROS activity

Figure 4.6 shows that 10-mM NAC pretreatment significantly antagonised HPc-induced potentiation of liver-specific metabolic function of hepatocytes co-cultured with HPc-MSCs. Hepatocytes co-cultured with NAC-pretreated HPc-MSCs secreted significantly less albumin, as compared to those with non-NAC-pretreated HPc-MSCs, from day 1 (HPc-Co *vs.* HPc+NAC-Co, 1.2 ± 0.3 *vs.* 0.9 ± 0.1 μg/10^6 hepatocytes; P <0.05) until day 7 (5.4 ± 0.3 *vs.* 4.5 ± 0.3 μg/10^6 hepatocytes; P <0.01). Moreover, hepatocytes co-cultured with NAC-pretreated HPc-MSCs synthesised significantly less urea, as compared to those with non-NAC-pretreated HPc-MSCs, from day 3 (32.8 ± 3.1 *vs.* 22.8 ± 2.2 μg/10^6 hepatocytes; P <0.05) until day 7 (40.8 ± 3.6 *vs.* 35.3 ± 3.3 μg/10^6 hepatocytes; P <0.01).

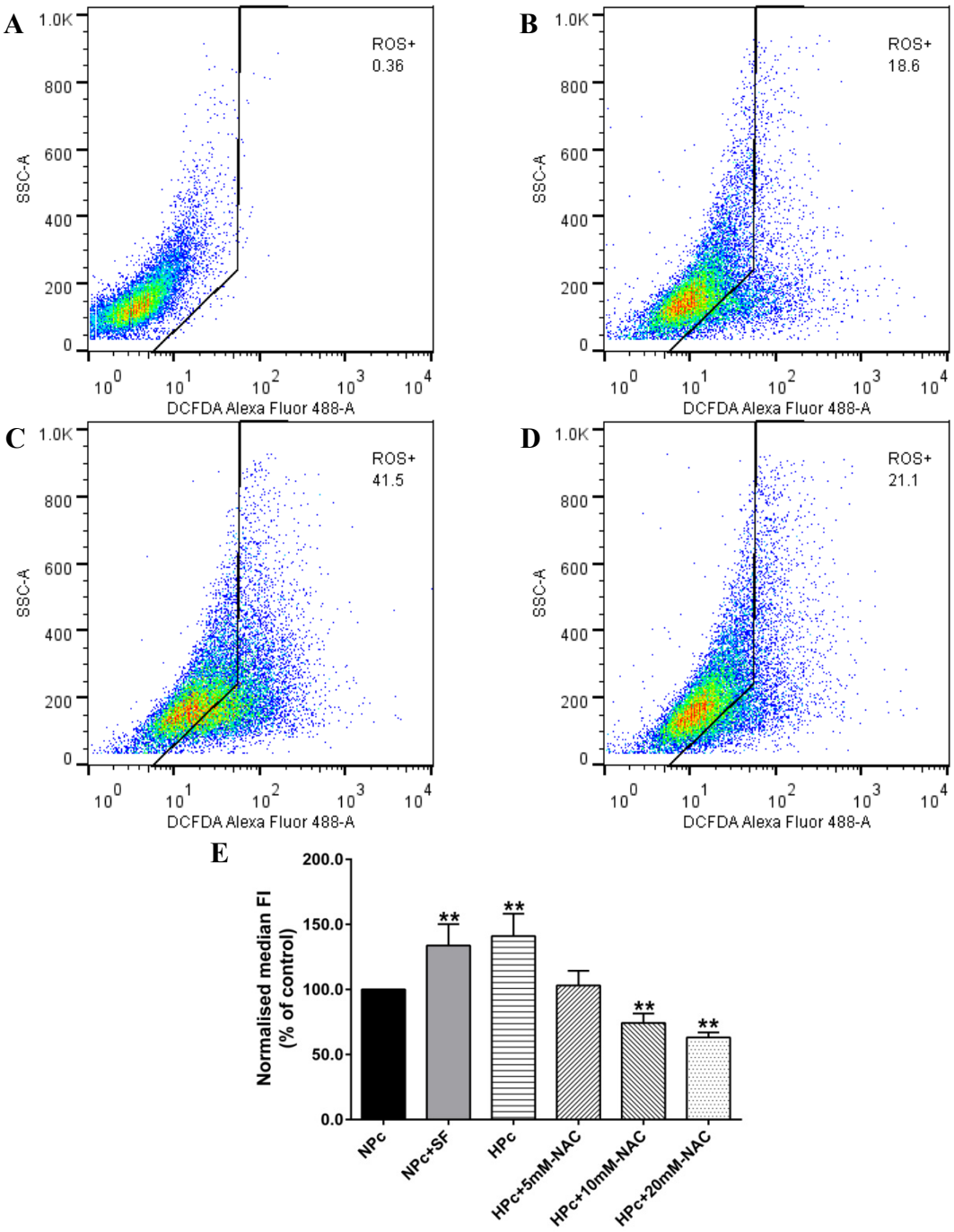

Figure 4.5 Effect of HPc and NAC pretreatment on intra-MSCs ROS activity as measured by flow cytometry with DCFDA: representative SSC-*versus*-DCFDA (intracellular ROS) FI scatter plots of unlabelled (A), NPc- (B), HPc- (C), and HPc+10-mM-NAC-pretreated (D) MSCs, and bar charts of normalised median FI (E) of intra-MSCs ROS activity. Serum deprivation and HPc significantly increased intra-MSCs activity as compared to NPc, while 10- and 20-mM NAC pretreatment significantly eliminated HPc-induced intra-MSCs ROS increase. All data were expressed as mean ± SD; **P* <0.05 and ***P* <0.01 *versus* NPc-MSCs (*n* = 6). SSC, side scattered; NPc, normoxia-preconditioned; SF, serum-free; HPc, hypoxia-preconditioned; NAC, N-acetylcysteine; nMean FI, normalised mean; nMedian, normalised median; FI, fluorescence intensity.

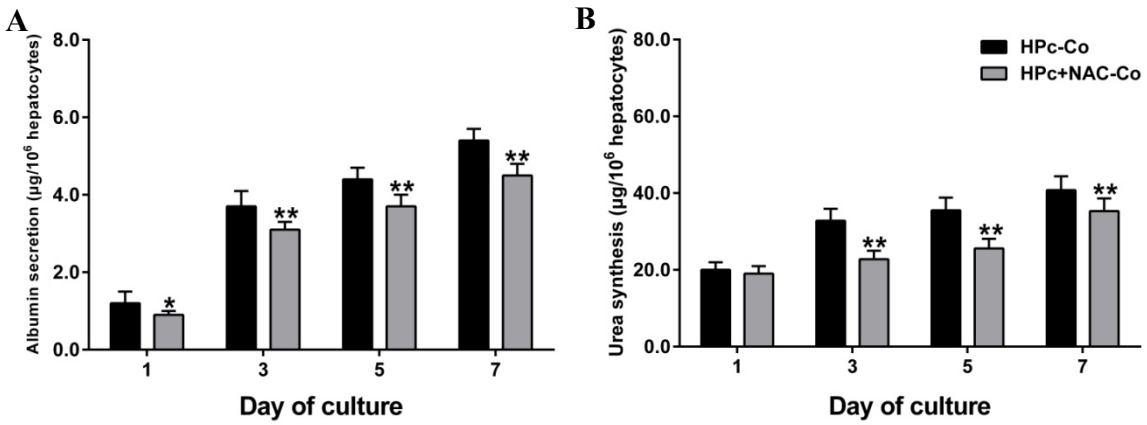

Figure 4.6 Albumin secretion (A) and urea synthesis (B) of hepatocytes co-cultured with HPc-, non-NAC-pretreated MSCs *versus* with HPc-, 10-mM NAC-pretreated MSCs. NAC pretreatment significantly antagonised HPc-induced potentiation of hepatotrophic effect on liver-specific metabolic function. All data were expressed as mean ± SD (error bar); **P* <0.05 and ***P* <0.01 *versus* HPc co-culture (*n* = 6). HPc, hypoxia-preconditioned; NAC, N-acetylcysteine; Co, co-culture.

4.3.5 *Potentiative effect of HPc on MSCs co-culture antiapoptotic effect*

4.3.5.1 HPc-potentiated co-culture antiapoptotic effect depends on intra-MSCs ROS activity

No CCK18 or CK18 release was detected in HPc-MSCs mono-culture. Direct co-culture with HPc-MSCs significantly further reduced CCK18 release from hepatocytes as compared to control NPc-MSCs co-culture (Figure 4.7A). Soluble CCK18 level remained significantly lower in hepatocytes co-cultured with HPc-MSCs than that in hepatocytes co-cultured with NPc-MSCs from day 1 (HPc-Co *vs.* NPc-Co, 14.5 ± 1.6 *vs.* 18.0 ± 1.4 U/10^6 hepatocytes; *P* <0.01) until day 5 (6.4 ± 0.9 *vs.* 9.2 ± 1.1 U/10^6 hepatocytes; *P* <0.01); however, 10-mM NAC pretreatment eliminated HPc-induced potentiation of MSCs antiapoptotic effect on co-cultured hepatocytes from day 1 (HPc+NAC-Co, 18.3 ± 1.4 U/10^6 hepatocytes; *P* <0.01) until day 5 (9.0 ± 10 U/10^6 hepatocytes; *P* <0.01).

Direct co-culture with HPc-MSCs also significantly further reduced CK18 release from hepatocytes, as compared to that with NPc-MSCs (Figure 4.7B), from day 1 (61.6 ± 4.8 *vs.* 91.2 ± 8.2 U/10^6 hepatocytes; *P*<0.01) until day 5 (61.8 ± 5.2 *vs.* 100.9 ± 8.1 U/10^6 hepatocytes; *P* <0.01); however, NAC pretreatment eliminated HPc-induced potentiation of MSCs prosurvival effect on co-cultured hepatocytes from day 3 (176.0 ± 16.8 U/10^6 hepatocytes; *P* <0.01) until day 5 (99.0 ± 8.6 U/10^6 hepatocytes; *P* <0.01).

As is shown in Figure 4.7C, CCK18/CK18 ratio remained similar between hepatocytes co-cultured with HPc-MSCs and hepatocytes co-cultured with NPc-MSC from day 1 (23.6% ± 2.6% *vs.* 20.2% ± 3.0%; *P* >0.05) until day 7 (1.7% ± 0.1% *vs.* 1.5% ± 0.1%; *P* >0.05). It was noted that NAC pretreatment of HPc-MSCs switched death mode of co-cultured hepatocytes from necrosis to apoptosis on day 1 (29.1% ± 2.4%; *P* <0.01).

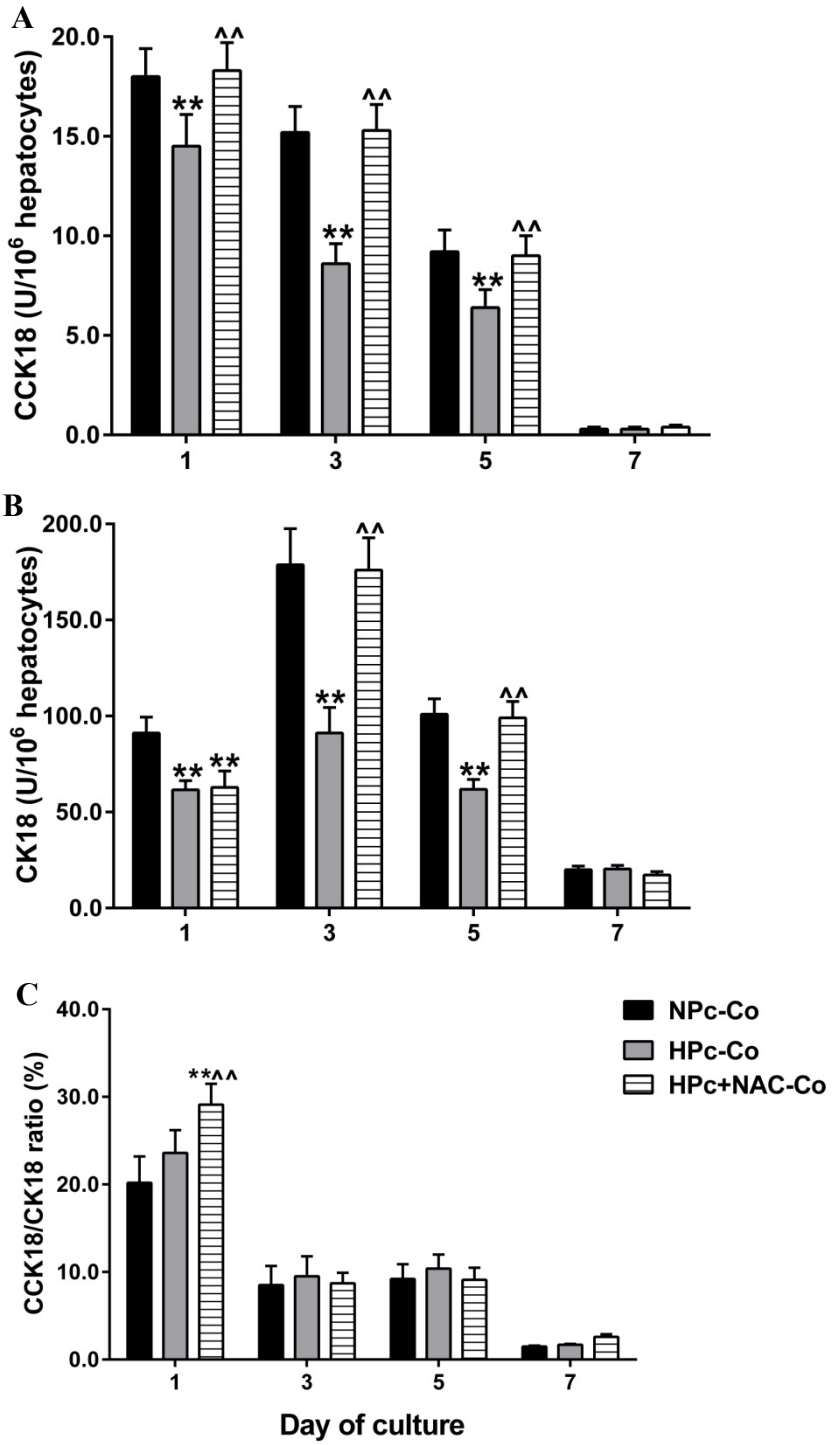

Figure 4.7 CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes co-cultured with NPc-MSCs *versus* with non-NAC-pretreated HPc-MSCs *versus* NAC-pretreated HPc-MSCs. HPc-MSCs co-culture significantly further reduced caspase-mediated apoptosis and total death of hepatocytes, and NAC pretreatment eliminated HP-potentiated MSCs co-culture antiapoptotic and prosurvival effects. All data were expressed as mean ± SD; ***P* <0.01 *versus* NPc-Co; ^^*P* <0.01 *versus* HPc-Co (*n* = 6). NPc, normoxia-preconditioned; HPc, hypoxia-preconditioned; Co, co-culture; NAC, N-acetylcysteine.

4.3.5.2 HPc does not potentiate MSCs paracrine contribution to co-culture antiapoptotic effect

MSCs indirect co-culture using Transwell plates Indirect co-culture with HPc-MSCs had no significant effect on hepatocyte CCK18 or CK18 release as compared to control NPc-MSCs indirect co-culture (Table 4.5). Soluble CCK18 level remained similar between hepatocytes indirectly co-cultured with HPc-MSCs and with NPc-MSCs from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes indirectly co-cultured with HPc-MSCs and with NPc-MSCs from day 1 until day 7. CCK18/CK18 ratio remained similar between hepatocytes indirectly co-cultured HPc-MSCs and with NPc-MSCs from day 1 until day 7.

MSCs co-culture CM HPc-MSCs co-culture CM had no significant effect on hepatocyte CCK18 or CK18 release from hepatocytes as compared to control NPc-MSCs co-culture CM (Table 4.6). Soluble CCK18 level remained similar between hepatocytes cultured with HPc- and NPc-MSCs co-culture CM from day 1 until day 7. Soluble CK18 level also remained similar between hepatocytes cultured with HPc- and NPc-MSCs co-culture CM from day 1 until day 7. CCK18/CK18 ratio remained similar between hepatocytes cultured with HPc- and NPc-MSCs co-culture CM from day 1 until day 7.

4.3.5.3 HPc potentiates MSCs co-culture hepatoprotective effect against staurosporine-induced cytotoxicity

Co-culture with HPc-MSCs significantly further reduced staurosporine-induced hepatocyte apoptosis (soluble CCK18 level) as compared to co-culture with NPc-MSCs (HPc *vs.* NPc, 21.6 ± 2.8 *vs.* 26.5 ± 2.5 U/10^6 hepatocytes, P <0.01; Figure 4.8A). However, HPc-MSCs co-culture had no significant effect on staurosporine-induced total cell death of hepatocytes as compared to NPc-MSCs co-culture (Figure 4.8B). However, HPc-MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis switch of hepatocyte death mode to a similar extent as compared to NPc-MSCs co-culture (Figure 4.8C).

Table 4.5 **CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes indirectly co-cultured with HPc-MSCs *versus* with NPc-MSCs**

	CCK18 (U/10^6 hepatocytes)				CK18 (U/10^6 hepatocytes)				CCK18/CK18 ratio (%)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
HPc-iCo	21.2 ± 2.6	22.7 ± 2.5	14.1 ± 1.8	1.4 ± 0.3	99.8 ± 11.7	375.0 ± 30.4	255.6 ± 28.9	45.9 ± 4.3	21.2 ± 2.4	6.1 ± 1.2	5.5 ± 1.1	3.1 ± 0.2
NPc-iCo	20.8 ± 2.4	23.2 ± 2.3	15.9 ± 1.7	1.6 ± 0.2	108.8 ± 12.4	390.0 ± 35.0	276.0 ± 33.0	50.4 ± 4.6	19.1 ± 2.8	5.9 ± 1.1	5.8 ± 1.3	3.2 ± 0.1

HPc, hypoxia-conditioned; NPc, normoxia-preconditioned; iCo-, indirect co-culture.

Table 4.6 **CCK18 release, CK18 release, and CCK18/CK18 ratio (mean ± SD) of hepatocytes cultured with HPc- *versus* NPc-MSCs co-culture CM**

	CCK18 (U/10^6 hepatocytes)				CK18 (U/10^6 hepatocytes)				CCK18/CK18 ratio (%)			
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
HPc-	22.1 ± 2.2	23.2 ± 2.1	17.1 ± 1.9	2.1 ± 0.3	112.2 ± 12.2	358.0 ± 31.1	290.0 ± 35.5	52.5 ± 4.5	19.7 ± 2.4	6.5 ± 1.2	5.9 ± 1.1	4.0 ± 0.3
NPc-	20.3 ± 2.1	22.5 ± 2.0	16.3 ± 1.8	2.0 ± 0.4	110.5 ± 12.5	360.0 ± 33.3	286.0 ± 31.7	54.1 ± 4.0	18.4 ± 2.0	6.3 ± 1.1	5.7 ± 1.0	3.7 ± 0.2

HPc, hypoxia-conditioned; NPc, normoxia-preconditioned.

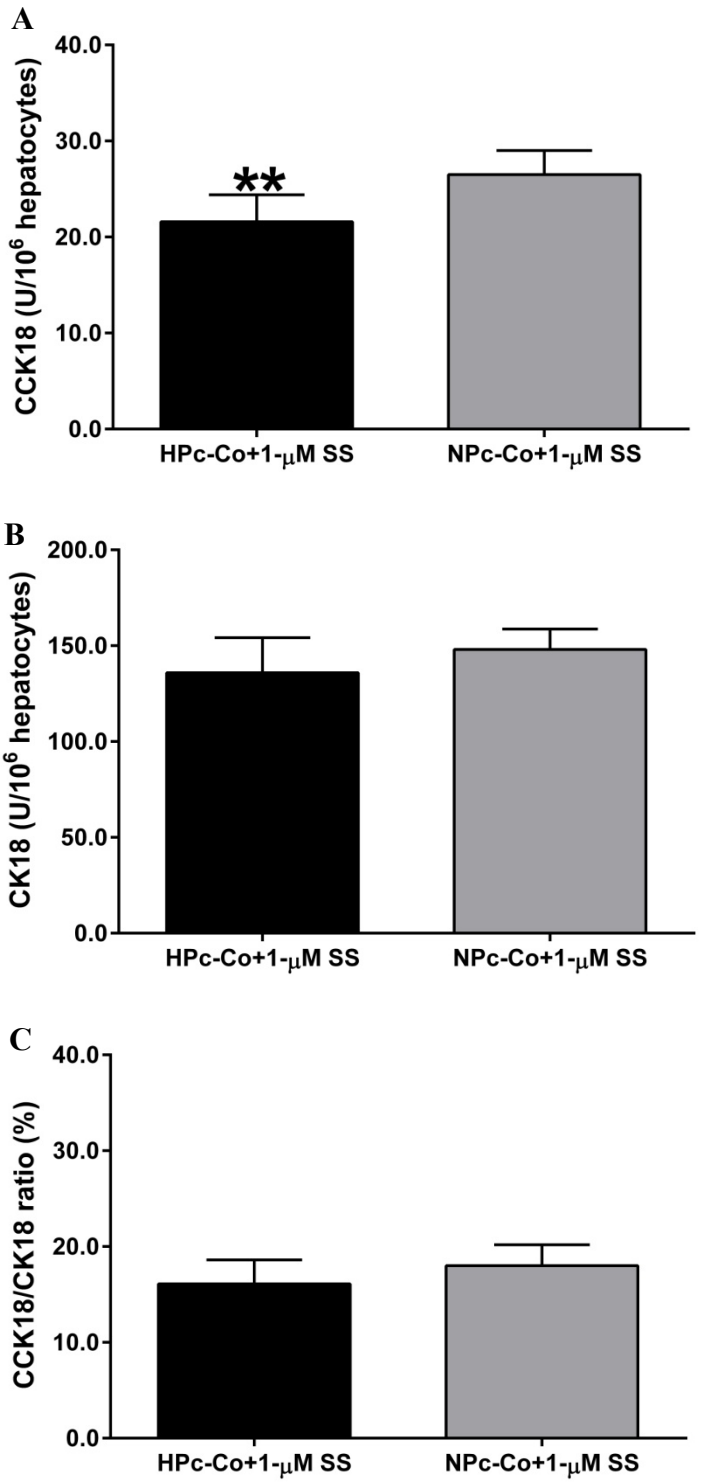

Figure 4.8 CCK18 release (A), CK18 release (B), and CCK18/CK18 ratio (C) of hepatocytes subjected to 1-μM staurosporine and co-cultured with HPc-MSCs *versus* with NPc-MSCs. HPc-MSCs co-culture significantly further reduced staurosporine-induced hepatocyte apoptosis and had no significant effect on total death of hepatocytes; HPc-MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis death mode switch to a similar extent as compared to NPc-MSCs co-culture. All data were expressed as mean ± SD; $^{**}P < 0.01$ *versus* control NPc-MSCs co-culture ($n = 6$). HPc, hypoxia-preconditioned; NPc, normoxia-preconditioned; Co, MSCs co-culture; SS, staurosporine.

4.4 Discussion

4.4.1 *Effects of HPc on MSCs*

MSCs are known to be highly sensitive to oxygen tension in the culture environment. Previous reports were controversial regarding hypoxic effects on MSCs, probably due to the variations in MSCs sources as well as hypoxic conditioning protocols. The variation in hypoxic effect on MSCs is most striking in cellular apoptosis: hypoxic conditioning was reported to be pro-apoptotic (Rasmussen *et al.*, 2011), anti-apoptotic (Zhang *et al.*, 2009), or without effect (Efimenko *et al.*, 2011). Severe hypoxia will distort the morphology and ultrastructure of MSCs, especially the mitochondria and nucleus; however, moderate hypoxic atmosphere favours human MSCs expansion *in vitro*. Hypoxia normally results in a smaller size of MSCs containing a less number of mitochondria, but hypoxia alone does not induce apoptosis in MSCs, but serum or glucose deprivation will cause marked apoptotic death in MSCs. In the present work, 24-hour 2% O_2 hypoxia had no cytotoxic effect on human AT-MSCs as shown by the morphological results consistent with a previous report (Fu *et al.*, 2011). It is generally accepted that HPc-MSCs exhibit a higher survival rate when subsequently subjected to hypoxia or hypoxia-reoxygenation, mainly by attenuating apoptosis in MSCs. Low-dose oxidative preconditioning can protect MSCs from cellular apoptosis induced by high-dose oxidative stress (Li *et al.*, 2009).

Hypoxia, as a major cause of mitochondrial oxidative stress, has a negative effect on mitochondrial metabolism. Hypoxia (1% O_2) decreases human BM-MSCs mitochondrial activity by inhibiting expression of respiratory enzymes and oxygen consumption; under a hypoxic condition, MSCs depends mainly on anaerobic glycolysis for energy supply, accompanied by upregulated expression of glycolytic enzymes and increased production of lactate acid. Hypoxia also activates cell-matrix contact of MSCs. Hypoxia under 2% O_2 upregulates expression of integrin subunits, including α1, α3, α5, α6, α11, β1, and β3, in human BM-MSCs (Saller *et al.*, 2012). The HPc protocol used in the present work did inhibit mitochondrial dehydrogenase activity as evidenced by decreased MTT activity but improved cellular attachment as shown by the increased SRB activity.

HPc is reported to positively affect the colony-forming potential and proliferation of BM- and UC-MSCs (Martin-Rendon *et al.*, 2007; Pilgaard *et al.*, 2009), while hypoxia-mimetic agents can inhibit the hypoxic effect on MSCs proliferation by inducing a G_1-phase cell cycle arrest (Zeng *et al.*, 2011). BM-MSCs cultured under 2% O_2 exhibit an advanced exponential growth phase and greater cell division kinetics (Dos Santos *et al.*, 2010). Physiological hypoxia (1%–3% O_2) enhances rat BM-MSCs proliferation, which manifests as increases in the ratio of S-phase cells, bromodeoxyuridine incorporation, and proliferating cell nuclear antigen expression, by upregulating phosphorylation of p38 MAPK and nuclear translocation of HIF-1α (Wang *et al.*, 2013). Hypoxic condition also enhances the propagation ability of human BM-MSCs possibly by maintaining telomere length (Tsai *et al.*, 2011). The ^{3}H-thymidine incorporation assay in the present work demonstrated that 24-hour of 2%-O_2 hypoxia promoted DNA synthesis in MSCs. The effect of hypoxia on overall protein synthesis was less reported in previous studies, although expression of

specific proteins was inhibited or activated in hypoxic MSCs (Abdollahi *et al.*, 2011). The ^{14}C-leucine incorporation assay in the present work showed that hypoxia slightly decreased overall protein synthesis. It is expected that MSCs synthesis of protein is inhibited under a hypoxic micro-environment as hypoxia switches the energy metabolism from oxidation phosphorylation to glycolysis; however, it is known that translation of protein generally requires a large amount of energy supply. It is likely that the stimulatory effect of hypoxia on MSCs protein synthesis will become evident after a longer time as reported in previous studies (Sengupta *et al.*, 2010).

4.4.2 *HPc potentiates trophic and protective effects of MSCs co-culture on hepatocytes by non-paracrine, ROS-dependent mechanisms*

Enhanced angiogenesis of HPc-MSCs has been well documented in literature, especially in the setting of myocardial, limb and cerebral ischaemia. HPc upregulates expression of various pro-survival and pro-angiogenic factors, especially angiopoietin-1, erythropoietin, and VEGF in MSCs, transplantation of which consequently enhances angiogenesis as well as morphological and functional restoration of the infarcted myocardium (Hu *et al.*, 2008). HPc also helps BM-MSCs to more effectively restore the blood flow in an experimental rat hind limb ischaemia model by improving revascularisation through activating HGF signalling (Rosová *et al.*, 2008). Moreover, HPc AT-MSCs also significantly increased the viability and decreased apoptotic death of co-cultured or co-transplanted neural stem cells by downregulating Bax signalling in an experimental rat spinal cord ischaemia model (Oh *et al.*, 2010). The present work demonstrated for the first time that HPc potentiated the hepatotrophic effect of MSCs on co-cultured hepatocytes with respect to liver-specific metabolic function *in vitro*.

Putative paracrine mechanisms are thought to primarily contribute to the potentiative effect of HPc on MSCs. HPc was reported to significantly upregulate expression of a large number of soluble cytokines and growth factors, such as HGF, VEGF, FGF2, IGF-1, and IL-6 in MSCs (Lam *et al.*, 2010). HGF and VEGF are two candidate factors that have been most frequently studied. HPc significantly increases the secretion of both HGF and VEGF from human BM-MSCs into the culture medium; concentrated HPc-MSCs conditioned medium favours the regeneration of neurons *in vivo* by limiting neuronal apoptosis resulting from experimental traumatic brain injury in rats, and further improves motor and cognitive function of the rat model (Chang *et al.*, 2013). Hsiao *et al.* (2013) reported that HPc enhanced paracrine angiogenic activity of human AT-MSCs through VEGF-A and angiogenin signalling pathways. HPc also increases human AT-MSCs secretion of VEGF and bFGF to the culture medium, and HPc-MSCs conditioned medium improves vitality and reduces apoptosis in human umbilical vein endothelial cells with enhanced tube formation *in vitro* (Liu *et al.*, 2013). Yu *et al.* (2013) recently reported an interesting study regarding BM-MSCs therapy for an experimental rat extensive hepatectomy model. NPc-MSCs did improve hepatocyte proliferation *in vivo* but not liver regeneration or animal survival, while HPc-MSCs significantly enhanced liver regeneration and animal survival. Activation of VEGF signalling in HPc-MSCs was thought to be

the underlying mechanism as expression of VEGF signalling was significantly upregulated and the improved therapeutic effects could be diminished by VEGF neutralisation antibody. However, the present work showed that HPc minimally potentiate paracrine contribution to trophic and antiapoptotic effects of MSCs on co-cultured human hepatocytes. It was possible that isolated human hepatocytes became unresponsive to HPc-potentiated MSCs release of soluble trophic and antiapoptotic factors. Therefore, HPc-induced potentiation of MSCs co-culture hepatotrophic and antiapoptotic effects may result primary from enhanced ECM construction and heterotypic MSC-hepatocyte communication.

Intracellular ROS is known to finely modulate biological activities of MSCs. Exposure to moderate hypoxia is known to immediately increase intracellular ROS production in human BM-MSCs (Busletta *et al.*, 2011), while pretreatment with NAC significantly inhibits increased ROS production and ameliorates oxidative stress associated cellular damages in hypoxic MSCs (Fan *et al.*, 2011). NAC pretreatment improves the antioxidant capacity of human MSCs to restore the reduction-oxidation balance by eliminating intracellular ROS, elevating intracellular glutathione level, and enhancing cellular adhesion when exposed to *in vitro* oxidative stress (Wang *et al.*, 2013). Oxidative stress resulting from low-level broadband visible light illumination was also found to stimulate the proliferative potential of BM-MSCs, in which production of ROS increased (Lipovsky *et al.*, 2013). These findings demonstrate that ROS is a major factor mediating the effects of oxidative stress, such as hypoxia and ion radiation, on cellular proliferation, survival/apoptosis, and attachment of MSCs *in vitro*.

The present work showed that scavenge of increased ROS reversed HPc-induced increases in synthesis of DNA and membrane proteins of human AT-MSCs, implying that ROS are involved in regulating DNA synthesis and cellular attachment. However, the paradox that addition of NAC further significantly reduced mitochondrial dehydrogenase activity and protein synthesis suggested the possibility that a pre-existing, relatively higher intracellular ROS level facilitates MSCs recovery from moderate hypoxia and resistance to subsequent hypoxia/reoxygenation-induced inhibitive effect on mitochondrial activity and protein synthesis of MSCs (Wang *et al.*, 2008). This also indicated the involvement of non-ROS-dependent mechanisms, such as HIF-1α, in HPc of MSCs. Upregulation of HIF-1α expression occurs at a late phase of HPc and mediates the cascades of hypoxic response (Busletta *et al.*, 2011). Overexpression of HIF-1α enhances the survivability, attachment, migration, ECM synthesis, osteogenic differentiation and energy metabolism of MSCs (Palomäki *et al.*, 2013).

The present work showed that HPc potentiated hepatotrophic and antiapoptotic effects of MSCs on co-cultured hepatocytes and NAC pretreatment diminished HPc-induced potentiative effects as evidenced by liver-specific metabolism and caspase-mediated hepatocyte apoptosis assays. Induction of ROS production accompanies phosphorylation of EGF receptor in human BM-MSCs, which can be antagonised by NAC treatment (Park *et al.*, 2013). Aged AT-MSCs show a relatively limited angiogenic capacity associated with significant downregulation of VEGF, placental growth factor, and HGF, while HPc significantly upregulated expression of these pro-angiogenic factors and

restored angiogenesis of aged AT-MSCs (Efimenko *et al.*, 2011). De Barros *et al.* (2013) also reported that aged human AT-MSCs had a relatively lower ROS level, while HPc could improve angiogenic capacity of aged MSCs; conversely NAC treatment eliminated HPc-induced potentiation of MSCs angiogenesis both *in vitro* and *in vivo*. It remains less investigated whether ROS also regulate ECM activity involved in trophic and antiapoptotic effects of MSCs on co-cultured hepatocytes. Chemically-induced oxidative stress was reported to upregulate expression of bone morphogenetic protein 2 and FGF2 in human AT-MSCs in an intra-MSCs ROS dependent manner (Moriyama *et al.*, 2012). These two cytokines are well known to actively participate in ECM formation and modification of bone and cartilages (Krawczak *et al.*, 2009).

4.4.3 *Conclusions*

In conclusion, as compared to NPc, HPc potentiated trophic effect of MSCs on co-cultured hepatocytes with respect to liver-specific metabolic function. HPc also enhanced antiapoptotic and protective effects of MSCs co-culture in an ROS-dependent manner as pretreatment with antioxidative NAC diminished HPc-induced potentiative effects. However, This potentiative effect might not result primarily from enhanced paracrine activities of MSCs regardless of the presence or absence of MSC-hepatocyte direct contact. It remains to be investigated whether HPc-induced potentiative effect on MSCs co-culture results from nonparacrine contribution of soluble factors and ECM secreted by MSCs by an ROS-dependent mechanism. Signalling pathways involved in apoptosis and survival of hepatocytes, such as caspases and BAX/BCL-2, will also be addressed in the next chapter.

CHAPTER 5 MECHANIMS OF MSCs CO-CULTURE HEPATOTROPHIC EFFECT AND HPC-INDUCED POTENTIATIVE EFFECT

5.1 Introduction

5.1.1 Contributive factors to hepatotrophic effect of MSCs co-culture

Trophic and protective effects of MSCs on co-cultured hepatocytes *in vitro* have been well documented in the present work and in previous studies. MSCs also have pronounced effects on hepatocyte proliferation, repair, and regeneration in physiological and pathological conditions *in vivo* (Esrefoglu, 2013). MSCs transplantation (Meier *et al.*, 2013) and co-transplantation with hepatocytes (Joshi *et al.*, 2012) are expected to be therapeutically effective for acute and chronic liver diseases. Putative mechanistic factors contributing to the hepatotrophic effects of MSCs co-culture *in vitro* mimic supportive factors for hepatocytes *in vivo*. These factors consist mainly of paracrine factors (soluble cytokines and growth factors), ECM, and heterotypic MSC-to-hepatocyte interaction (Figure 5.1; Gómez-Aristizábal *et al.*, 2009).

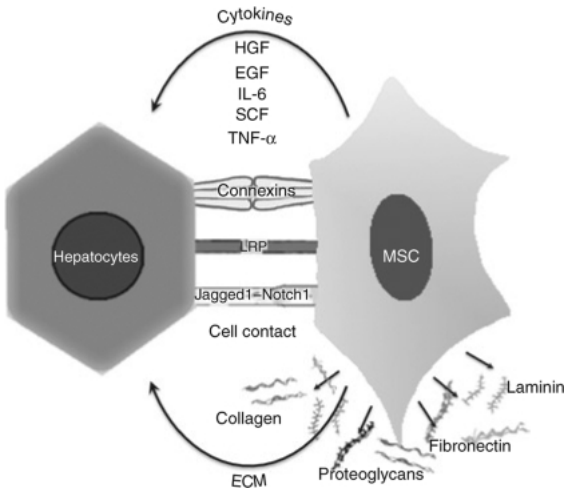

Figure 5.1 Putative mechanisms of trophic effect of MSCs on co-cultured hepatocytes, including soluble cytokines, ECM, and direct cell contact (Gómez-Aristizábal *et al.*, Mol Ther, 2009).

5.1.1.1 Soluble factors in MSCs co-culture

Soluble factors contributing to the trophic effect of MSCs on co-cultured epithelial cells and other cell lines, such as cardiomyocytes, hepatocytes, and islet cells, have been relatively well studied in the current literature. Injection with CM from Akt-overexpressing MSCs enriched with VEGF, FGF-2, HGF, IGF-I, and TB4 significantly shrank infarction size and improved ventricular function (Gnecchi *et al.*, 2006). Gu and his colleagues (2009[a]) reported that MSCs co-culture with hepatocytes significantly increased secretion of IL-6 rather than TGF-α or TNF-α; conversely IL-6 neutralisation significantly diminished the trophic effects of MSCs co-culture CM on liver-specific metabolism. Moreover, MSCs-derived soluble factors have supportive effect on transplanted cells

after transplantation *in vivo*. Park *et al.* (2010) demonstrated that HGF, IL-6, TGF-β, and VEGF-A were the trophic molecules of human BM- and UC-MSCs enhancing survival, function, and angiogenesis of isolated islets after transplantation. Overexpression of VEGF and HGF in MSCs significantly increased cardiomyocytes survival and peri-infarct vessel density in the border zone of acute myocardial infarction (Deuse *et al.*, 2009). MSCs CM also protects hepatocytes from CCl_4-induced apoptosis as mediated by IL-6 signalling and consequent activation of fibroblast-like-protein 1 (Xagorari *et al.*, 2013). Preliminary analysis of MSCs secretome using the proteomics technique showed out of all chemokines only heparin-bound eluent was effective for treating fulminant hepatic failure (Parekkadan *et al.*, 2007). Interestingly, trophic factors may have an autocrine regulative effect on MSCs. *Ex vivo* treatment of human BM-MSCs with ischaemic rat brain extract containing high levels of brain-derived neurotrophic factor, VEGF, and HGF results in a further increase in production of these growth factors (Choi *et al.*, 2010). The physiological activities of lipid microvesicles released from MSCs have been recently discovered but not well understood. Generally these microvesicles play a sophisticated role in transporting proteins, lipids, and RNAs to neighbouring cells (Baglio *et al.*, 2012).

5.1.1.2 ECM in MSCs co-culture

ECM is historically known to play an essential role in maintaining and regulating hepatocyte survival, proliferation, maturation, regeneration, and metabolism (Depreter *et al.*, 2000). ECM supports hepatocyte directly through cell-to-ECM interaction or indirectly through modulating release of growth factors, such as HGF (Schuppan *et al.*, 1998) and bFGF (Sakakura *et al.*, 1999). Minimal self-deposition of ECM may result in deterioration of cell survival and metabolic function in hepatocyte mono-culture due to the absence of cell-to-matrix interaction (Thomas *et al.*, 2005); interruption of cellular attachment to ECM, such as laminin, fibronectin, and collagen type I and V by neutralising β1-integrin results in marked apoptosis of hepatocytes (Pinkse *et al.*, 2004). Engineered bioscaffolds containing synthetic ECM, such as xyloglucan (Seo *et al.*, 2005) have been developed to maintain and support hepatocytes both *in vitro* and *in vivo*. Co-culture of rabbit chondrocytes with allogeneic BM-MSCs at an optimal ratio (chondrocytes:MSC, 2:1) improves expression of type II collagen and aggrecan, two major components of cartilaginous ECM (Qing *et al.*, 2011). MSCs co-encapsulation was also reported to synergistically enhance insulin secretion of pancreatic islets with ECM by upregulating expression of insulin I/II, glucagon, somatostatin, and pancreatic and duodenal HOX 1, and downregulating expression of CK19 and vimentin (Davis *et al.*, 2012). Gu and his colleague (2009[c]) reported that mono-cultured hepatocytes deposited minimal ECM, such as fibronectin, laminin, and collagen type I, III, and V, while MSCs co-culture secreted fibronectin and collagen type I, as well as some laminin and collagen type V; further siRNA knockdown experiments validated that inhibition of fibronectin, laminin, and collagen type I and V compromised the hepatotrophic effect of MSCs on co-cultured hepatocytes with respect to liver-specific metabolic function. Interaction between MSCs and ECM also modulates capillary morphogenesis of vascular endothelial cells both *in vitro* and *in vivo*, suggesting the role of MSCs in

neoangiogenesis in addition to paracrine activity (Kniazeva *et al.*, 2011). It is noted that MSCs have a biphasic modulatory effect on ECM formation of the liver (Zhao *et al.*, 2012). Li *et al.* (2013) reported that BM-MSCs transplantation significantly decreased hydroxyproline content and collagen accumulation in an experimental liver fibrosis model, accompanied by upregulated expression of HGF, IL-10, VEGF, and MMP-9.

5.1.1.3 Cell-to-cell contact in MSCs co-culture

Epithelial-to-mesenchymal interaction (EMI) plays a paramount role in reciprocal regulation of biological activities involved in development and regeneration of the liver. Moreover, liver MSCs also promotes differentiation and maturation of hepatic progenitor cells by direct cell-to-cell contact (Hoppo *et al.*, 2004; Ito *et al.*, 2013). The contribution of cell-to-cell contact to trophic effect of MSCs co-culture is evidenced by the fact that direct contact co-culture is superior to indirect non-contact co-culture and MSCs-derived CM in the present work and previous studies. Jung *et al.* (2011) reported that contact between MSCs and islet cells was a major factor favouring survival, morphology maintenance, and insulin release of pancreatic islets in the presence of synergic regulation of inflammatory cytokine production. Gómez-Aristizábal and Davies (2012) also reported that human UC perivascular cells contact co-culture significantly improved ureagenesis of human hepatocytes as compared to non-contact co-culture. EMI is modulated by multiple growth factors, such as EGF, TGF-α, HGF, TGF-β, bFGF, platelet-derived growth factor β, and IGF-1; out of these factors IGF-1 is the key to EMI during gastric mucosal healing (Watanabe *et al.*, 2000). This interaction is also modulated by membrane-associated ligand/receptor. The Notch signalling pathway is a highly conservative cellular signalling system regulating cell-to-cell contact and involved in remodelling liver progenitor cell niche in the setting of liver fibrosis (Spee *et al.*, 2010). Human BM-MSCs expresses a high level of Notch ligand on contact with rat neural stem cells, which highly express the receptor NOTCH1 (Robinson *et al.*, 2011). Moreover, human UC perivascular cells in co-culture with rat hepatocytes exhibited marked upregulation of HGF and Jagged1 (a ligand of NOTCH1 receptor) expression, suggesting the role of Notch-Jagged1 interaction in maintaining functional polarity of hepatocytes (Gómez-Aristizábal and Davies, 2013). Liver-regulating protein, which is widely expressed by BM-MSCs, hepatocytes, and liver NPCs, is thought to partially contribute to hepatotrophic effect of MSCs co-culture; blocking antibody against LRP will diminish the trophic effect of MSCs on co-cultured hepatocytes (Corlu *et al.*, 1997). Conversely direct contact with distinct differentiated cells also plays a critical role in determining MSCs fate. Wang *et al.* (2006) reported that direct contact co-culture with rat cardiomyocytes rather than indirect non-contact co-culture or culture with CM drove rat BM-MSCs differentiation into cardiomyocytes or smooth muscle cells.

5.1.2 HPc-induced potentiative effect

5.1.2.1 HPc potentiates paracrine activity

MSCs exhibit a potentiated paracrine activity in response to hypoxia (Das *et al.*, 2010), and HPc-potentiated paracrine effect of MSCs can improve the therapeutic benefit of MSCs transplantation (Cheng and Yau, 2008). Hypoxia triggers activation of Akt signalling pathway and consequently upregulates expression of genes encoding VEGF, FGF-2, HGF, IGF-1, and thymosin β4, enriched with which MSCs CM results in marked reduction of cardiomyocytes apoptosis (Gnecchi *et al.*, 2006). Hypoxic challenge was reported to upregulate expression of MSC VEGF, FGF2, HGF, and IGF-1 by an ERK- and JNK-independent, NFκB-dependent, mechanism (Crisostomo *et al.*, 2008). Hypoxic exposure also significantly increased secretion of VEGF, HGF, and bFGF, which depends on HIF, a pivotal signalling factor in hypoxic response (Tamama *et al.*, 2011). After HPc, rat BM-MSCs exhibit significantly upregulated expression of pro-survival and pro-angiogenic factors, such as HIF-1, angiopoietin-1, VEGF and its receptor, Flk-1, and erythropoietin (Hu *et al.*, 2008). HPc-MSCs CM shows a significantly greater protective effect on cardiomyocytes deprived of serum under 1%-O_2 hypoxia as compared to NPc counterpart (Fidelis-de-Oliveira *et al.*, 2012). HPc-MSCs enhanced skeletal muscle regeneration, to a greater extent as compared to NPc-MSCs, by improving blood flow and vascular formation in an experimental rat hind limb ischaemia model; the putative mechanism might be activation of *Wnt4* gene (Leroux *et al.*, 2010), encoding a secreted protein regulating myogenic proliferation. HPc also potentiates neurotrophic effects of rat BM-MSCs by upregulating expression of a series of trophic and growth factors, including brain- and glial cell-derived neurotrophic factor, VEGF and its receptor, erythropoietin and its receptor, SDF-1, and CXC chemokine receptor 4 (Wei *et al.*, 2012). These HPc-potentiated paracrine mechanisms are likely to offer additional benefits with respect to neovascularisation and progenitor cell recruitment if transplanted in vivo. HPc increases secretion of angiogenic VEGF and bFGF from AT-MSCs, by which HPc-MSCs CM improves survival and tube formation of human umbilical vein endothelial cells (Liu *et al.*, 2013). HPc stimulates secretion of proangiogenic and mitogenic factors and improved MSCs chemotaxis; upregulation of SFDF-1α expression in the ischaemic tissue selectively recruited HPc- rather than NPc-MSCs by a CXC receptor (CXCR) 7-independent, CXCR4-dependent manner (Liu *et al.*, 2012).

5.1.2.2 HPc enhances formation and organisation of ECM

Microenvironmental oxygen tension finely modulates chondrogenesis and osteogenesis of MSCs by switching on and off MSCs formation of ECM. Hypoxic conditions stimulate MSCs to deposit multiple components of ECM and adhesion molecules. A previous genomic study demonstrated that HPc had a late-phase upregulative effect on expression of genes involved in ECM by up to 60 folds (Basciano *et al.*, 2011). Hypoxia (5% O_2) rather than normoxia (21% O_2) in combination with TGF-β3 can induce chondrogenic differentiation of foetal synovium-derived MSCs in a serum free culture (Li *et al.*, 2011). Expression of MMP-2, collagen type II and XI, aggrecan, and integrins α2 and β3 is significantly upregulated in rat MSCs subjected to TGF-β1 and

2%-O_2 hypoxia along with phosphorylation of MAPK/ERK1/2 signalling pathway (Risbud *et al.*, 2004). IL-1β has an inhibitory effect on chondrogenesis, while hypoxic condition can reverse the inhibitory effects of IL-1β on BM-MSCs deposition of ECM (Felka *et al.*, 2009). Stimulatory effect of hypoxia on MSCs deposition of ECM can also be maintained after transplantation *in vivo*. Feng *et al.* (2011) reported that HPc in combination with TGF-β1 drove rabbit MSCs differentiation towards a nucleus pulposus-like phenotype *in vitro*, as evidenced by significantly upregulated expression of aggrecan, collagen type II, Sox-9, glycosaminoglycan, and HIF-1α; subcutaneous implantation experiment showed that HPc facilitated MSCs-scaffold construct to maintain chondrial morphology and prevent secondary calcification. Oxygen tension tunes MMPs and tissue inhibitor of MMPs in a differential manner; low oxygen tension inhibits expression of MMP-13 and tissue inhibitor of MMP-1 involved in ECM remodelling and vascular invasion, but has significant effects on expression of MMP-2, an enzyme involved in cell migration, in human MSCs (Raheja *et al.*, 2010). More interestingly, MSCs have a beneficial regulative effect on ECM remodelling in response to hypoxia. NPc- or HPc-MSCs co-culture downregulates expression of MMP and upregulates expression of tissue inhibitor of MMP-1 in cardiac fibroblasts subject to severe hypoxia (0.5% O_2) mediated by erythropoietin and its receptor as well as ERK1/2 signalling pathway (Wang *et al.*, 2011).

5.1.2.3 HPc potentially augments cell-to-cell contact

Effect of hypoxia on interaction inbetween MSCs and between MSCs and other cell lines is rarely reported in literature. Connexins are a family of structurally related transmembrane proteins that assemble the gap junction and serve as the electrical coupling in the neuromuscular tissue. Long-term hypoxic culture of human MSCs significantly upregulates expression of connexin 43 (Cx43), a connexin family member mainly found in myocardium, along with enhanced ECM formation. The biological role of Cx43 is not well understood; knockdown of Cx43 will reduce cardiomyocyte survival and diminish protective effect of IGF-1 on HSCs (Lu *et al.*, 2009). Overexpression of Cx43 significantly upregulates expression of Bcl-2, downregulates expression of Bax, increases phosphorylation of Akt signalling pathway in MSCs; Cx43-overexpressing MSCs shows an enhanced survival in infarcted myocardium (Wang *et al.*, 2010). Enhanced expression of Cx43 in response to hypoxia accompanies expression of prosurvival and proangiogenic factors in MSCs (Chacko *et al.*, 2010). Integrins are another collection of transmembrane receptors mediating adhesion between a cell and the neighbouring cells or ECM and transducing signals that regulate a number of biological activities, such as cellular survival, division, growth, differentiation, and apoptosis (Docheva *et al.*, 2007). Overexpression of integrin-linked kinase enhances attachment of hypoxic MSCs to ischaemic myocardium (Song *et al.*, 2009). It remains to be investigated whether hypoxia augments heterotypical interaction of MSCs with epithelial and other cells and how this augmented interaction improves supportive effect of MSCs.

5.1.3 Chapter objectives

- *Contribution of TNF-α and TGF-β1*

To investigate whether autocrine TNF-α activity of hepatocytes and autocrine TGF-β1 activity of MSCs mediate hepatotrophic effect of MSCs and potentiative effect of HPc induction.

- *Contribution of collagen*

To investigate whether extracellular collagen mediates hepatotrophic effect of MSCs and potentiative effect of HPc induction.

- *Pro- and antiapoptosis-associated gene expression analysis*

To characterise expression profiles of pro- and antiapoptosis-associated genes, such as caspase and BAX/BCL-2 signalling pathways, in MSCs co-culture and HPc co-culture as compared to those in mono-cultured hepatocytes.

5.2 Materials and methods

5.2.1 Cell culture protocols

5.2.1.1 Subculture and HPc of AT-MSCs and isolation of hepatocytes

Human AT-MSCs were subcultured as described in ***Section 2.2, Subculture of MSCs***. P6–8 MSCs were subjected to 2%-O_2 hypoxia (HPc-MSCs) or 20%-O_2 normoxia (NPc-MSCs) for 24 h as described in *Section 4.2.3, Optimisation of HPc*. Non-steatotic donor liver tissues were processed as described in ***Section 2.3, Primary Harvest of Human Hepatocytes***. Batches of hepatocytes with a viability of over 60% on trypan blue exclusion were used for experiments.

5.2.1.2 Direct co-culture of hepatocytes with MSCs

Fresh hepatocytes were co-cultured with HPc- *versus* NPc-MSCs as described in *Section 4.2.4.1*, *Direct co-culture of hepatocytes with MSC*. The seeding density of MSCs was 20,000 viable cells per cm^2, and that of hepatocytes was 50,000 viable cells per cm^2, at a MSC:hepatocyte ratio of 1:2.5. Mono-cultured hepatocytes were used as control, and mono-cultured HPc-/NPc-MSCs were used as blank controls.

5.2.1.3 Indirect co-culture of hepatocytes with MSCs

Hepatocytes were indirectly co-cultured with HPc- *versus* NPc-MSCs using Transwell® Permeable Supports, as described in *Section 4.2.4.2*, *Indirect co-culture of hepatocytes with MSCs*, to investigate soluble factors underlying the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction.

The culture media were collected 24 h after culture. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for assays. Cell cultures were rinsed with one-wash PBS and cryopreserved at −80°C for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.

5.2.2 Pretreatment experiments

5.2.2.1 NAC antagonisation experiment

HPc-MSCs were pretreated with 10-mM NAC as described in *Section 4.2.6.1*, *Optimisation of N-acetylcysteine concentration*. MSCs cultures were rinsed with one wash of PBS, and fresh hepatocytes were co-cultured with NAC-treated *versus* non-treated HPc-MSCs as described in *Section 4.2.4.1*, *Direct co-culture of hepatocytes with MSC*.

5.2.2.2 Staurosporine cytotoxicity experiment

Fresh hepatocytes were pre-treated with 1-μM staurosporine and co-cultured with NPc-, HPc-, and NAC-pretreated HPc-MSCs for 24 h, as controlled by staurosporine-treated mono-cultured hepatocytes.

5.2.2.3 TNF-α ELISA and neutralisation experiment

TNF-α ELISA The Quantikine® human TNF-α ELISA immunoassay kit (R&D Systems Europe, Ltd., Abingdon, UK) with the solid-phase sandwich enzyme immunoassay technique was used to quantitate soluble TNF-α level in cell cultures. The 96-well (12 strips of 8 wells) polystyrene microplate is pre-coated with a mouse monoclonal antibody against human TNF-α. All reagents were brought to room temperature 20 min prior to use. TNF-α standard was reconstituted with deionised water to prepare a stock solution of 10,000 pg/mL 15 min prior to dilution, and was serially diluted with the diluted calibrator diluent RD6-35 (animal serum) to give the standards 1,000, 500, 250, 125, 62.5, 31.2, 15.6 pg/mL, whilst the calibrator diluent was used as the zero standard (0 pg/mL). Cell culture supernates were thawed at room temperature and centrifuged at 1,500 rpm for 1 min to pellet any cell debris or particulates. The assay diluent RD1F (buffered protein base), 50 µL per well, was added, and the standards, samples, and controls were incubated in the assigned wells, 200 µL per well, at room temperature for 2 h. The wells were rinsed with four washes of detergent buffer. The bound TNF-α was detected by TNF-α conjugate (polyclonal antibody against TNF-α conjugated to HRP), 100 µL per well, at room temperature for 60 min. The HRP detection antibody was removed, and the wells were rinsed with four washes of detergent buffer. The substrate solution containing stabilised hydrogen peroxide and stabilised chromogen TMB in an equal volume, 200 µL per well, was added, and the plate was incubated in the dark and at room temperature for 20 min. The enzymatic colour reaction was stopped by adding 2 N sulphuric acid, 50 µL per well. The OD was measured at 450 nm using the microplate reader. The TNF-α concentration (pg/mL) in each sample was determined using the TNF-α standard curve, and the reading of background hepatocyte culture medium (blank control) was subtracted from that of mono- or co-cultured hepatocytes or MSCs to obtain the colorimetric OD of hepatocyte or MSCs culture *per se*.

TNF-α neutralisation experiment TNF-α neutralisation experiment was performed to investigate proapoptotic effect of TNF-α on mono-cultured hepatocytes. Human TNF-α affinity purified polyclonal antibody was purchased from R&D Systems Europe Ltd. (Abingdon, United Kingdom) and reconstituted at 1 mg/mL in sterile PBS. Freshly isolated primary human hepatocytes were seeded onto collagen-precoated 96-well plates at a density of 50,000 viable cells per cm^2 for a 24-h pre-incubation. The culture media were replaced by fresh hepatocyte culture media containing 0× (control), 1× (10 µg/mL), 2.5×, 5×, 10×, and 20× TNF-α neutralisation antibody for further 24-h mono-culture.

5.2.2.4 TGF-β ELISA and neutralisation experiment

TGF-β1 ELISA The Quantikine® human TGF-β1 ELISA immunoassay kit (R&D Systems Europe, Ltd., Abingdon, UK) with the solid-phase sandwich enzyme immunoassay technique was used to quantitate soluble TGF-β1 level in cell cultures. The 96-well (12 strips of 8 wells) polystyrene microplate is pre-coated with a mouse monoclonal antibody against human TGF-β1. All reagents were brought to room temperature 20 min prior to use. TGF-β1 standard was reconstituted with deionised water to prepare a stock solution of 2,000 pg/mL 15 min prior to dilution, and was

serially diluted with the diluted calibrator diluent RD5-53 (buffered protein base) to give the standards 1,000, 500, 250, 125, 62.5, 31.2, 15.6 pg/mL, whilst the calibrator diluent was used as the zero standard (0 pg/mL). Cell culture supernates were thawed at room temperature and centrifuged at 1,500 rpm for 1 min to pellet any cell debris or particulates. TGF-β1 is generally secreted as a latent form to the cell culture medium and becomes immunoreactive following acid activation and neutralisation. Briefly, the cell culture supernate (100 μL) was incubated with 1-N hydrochloride acid (20 μL; Sigma-Aldrich, St Louis, MO, USA) at room temperature for 10 min, and the acidified sample was neutralised by adding 1.2-N hydroxyl peroxide (20 μL; Sigma-Aldrich, St Louis, MO, USA) containing 0.5-M HEPES to a pH value of 7.2−7.6. The assay diluent RD1-21 (buffered protein solution), 50 μL per well, was added, and the standards, activated samples (diluted in 1.4 folds), and controls were incubated in the assigned wells, 50 μL per well, at room temperature for 2 h. The wells were rinsed with four washes of detergent buffer. The bound TGF-β1 was detected by TGF-β1 conjugate (polyclonal antibody against TGF-β1 conjugated to HRP), 100 μL per well, at room temperature for 2 h. The HRP detection antibody was removed, and the wells were washed with were rinsed with four washes of detergent buffer. The substrate solution containing stabilised hydrogen peroxide and stabilised chromogen TMB in an equal volume, 100 μL per well, was added, and the plate was incubated in the dark and at room temperature for 30 min. The enzymatic colour reaction was stopped by adding hydrochloride acid solution, 100 μL per well. The OD was measured at 450 nm using the microplate reader. The TGF-β1 concentration (pg/mL) in each sample was determined using the TGF-β1 standard curve, and the reading of background hepatocyte culture medium (blank control) was subtracted from that of mono- or co-cultured hepatocytes or MSCs to obtain the colorimetric OD of hepatocyte or MSCs culture *per se*.

TGF-β neutralisation experiment TGF-β1 neutralisation experiment was performed to investigate whether soluble TGF-β1 secreted by MSCs contributed to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. Human TGF-β1 affinity purified polyclonal antibody was purchased from R&D Systems Europe Ltd. (Abingdon, United Kingdom) and reconstituted at 1 mg/mL in sterile PBS. P4−6 human AT-MSCs were seeded onto collagen-precoated 96-well plates at a density of 20,000 viable cells per cm^2 for a 24-h pre-incubation. The culture media were replaced by fresh hepatocyte culture media containing 0× (control), 1× (1 μg/mL), 2.5×, 5×, 10×, and 20× TGF-β1 neutralisation antibody. Mono-cultured MSCs were further subjected to HPc *versus* NPc for 24 h, and fresh hepatocytes were co-cultured with HPc- and NPc-MSCs for additional 24 h as described in *Section 4.2.4.1*, *Direct co-culture of hepatocytes with MSC*.

5.2.2.5 Collagen assay and inhibition experiment

Cellular collagen assay Cellular collagen was semi-quantitated using the colorimetric assay with picro-sirius red (PSR) staining. PSR specifically binds to hydroxyproline residuals enriched in collagen (up to 14% by weight). PSR was gifted by Dr Qihe Xu (Department of Renal Medicine, King's College London) and reconstituted at 0.1% in saturated aqueous solution of picric acid (Sigma-Aldrich, St Louis, MO, USA). Cell cultures in chamber slides (Thermo Scientific Nunc,

Loughborough, UK) or 96-well plates were fixed in methanol (Sigma-Aldrich, St Louis, MO, USA) at -20°C overnight, and rinsed with two washes of PBS for 5 min. Cell cultures were stained in 0.1% PSR solution at room temperature for 4 h. The staining solution was discarded, and cell cultures were rinsed with three washes of 0.1% acetic acid for 5 min. PSR-stained cell cultures in chamber slides were dehydrated in three washes of absolute ethanol for 5 min, permeabilised in three washes of xylene for 10 min, and mounted with coverslips for light photomicrography. PSR-stained cell cultures in 96-well plates were eluted in 0.1 N sodium hydroxide (Sigma-Aldrich, St Louis, MO, USA), 200 µL per well, for 1 h for spectrophotometry. The OD was measured at 540 nm using the microplate reader.

Soluble and extracellular collagen assays Soluble and extracellular collagen was quantitated using the colorimetric assay with the Sircol™ soluble collagen assay kit (Biocolor, Carrickfergus, UK). Fresh (blank control) and thawed culture media, 1 mL per vial, were transferred into 1.5-mL low-protein-binding Eppendorf tubes (Eppendorf UK Limited, Stevenage, UK). The ice-cold isolation and concentration reagent containing Tris-HCl buffered polyethylene glycol (pH = 7.6) was added, 200 µL per vial, for an overnight incubation at 4°C. The tubes were centrifuged to pellet hydrated collagen at 12,000 rpm for 10 min, and the supernatant was removed using a 1-mL micropipette. The Sircol dye reagent containing PSR was added, 1 mL per vial, and the tubes were gently shaken for 30 min. The tubes were centrifuged to pellet the collagen-dye complex at 12,000 rpm for 10 min, and the supernatant was removed using a 1-mL micropipette. The ice-cold acid-salt wash reagent containing acetic acid, sodium chloride, and surfactants was gently layered on the collagen-dye pellet, 750 µL per vial, to remove unbound dye. The tubes were centrifuged to pellet the bound collagen-dye complex at 12,000 rpm for 10 min, and the supernatant was removed using a 1-mL micropipette. The alkali reagent containing 0.5-M sodium hydroxide was added, 250 µL per vial, to release the bound collagen-dye into the solution for 5 min. Samples were transferred to assigned wells of a clear-bottom 96-well plate, 200 µL per well. Extracellular collagen was solubilised by overnight incubation with 0.1 mg/mL pepsin (Sigma-Aldrich, St Louis, MO, USA) in 0.5-M acetic acid at 4°C. The acid extracts were neutralised by adding the acid neutralising reagent, 1 mL per vial, containing Tris-HCl buffered sodium hydroxide. Extracellular collagen was further stained using the same protocol, and collagen standards 0, 5, 10, and 15 µg reconstituted in 100-µL, 0.5-M acetic acid were also stained using the same protocol to produce the standard curve. The OD was measured at 540 nm using the microplate reader. The collagen content in each sample was determined using the collagen standard curve, and the reading of background hepatocyte culture medium (blank control) was subtracted from that of mono- or co-cultured hepatocytes or MSCs to obtain the colorimetric OD of hepatocyte or MSCs culture *per se*. The collagen content was normalised to that (mg) of 1 million hepatocytes or MSCs.

Collagen inhibition experiment N-(methylamino)isobutyric acid (MaIBA) is a competitive inhibitor of the neutral amino acid transport A system in collagen synthesis. HPc- and NPc-MSCs were subjected to 0-, 0.1-, 0.5-, 1-, 2.5-, 5-, 10-, 20-mM MaIBA (Sigma-Aldrich, St Louis, MO, USA) for 24 h. Cytotoxic effect of MaIBA on MSCs was evaluated using MTT and SRB

attachment assays as described in MTT assay was performed as described in ***Section 2.5*, *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Colorimetric Assay*** and ***Section 2.6***, ***Sulforhodamine B Colorimetric Assay***. Extracellular collagen deposit of MaIBA-treated HPc- and NPc-MSCs was determined using the Sircol™ soluble collagen assay kit as described above. The least concentration of MaIBA that resulted in significant reduction in extracellular collagen deposit of HPc- and NPc-MSCs was used for further inhibition experiment. MaIBA-treated *versus* non-treated HPc- and NPc-MSCs were co-cultured with hepatocytes for 24 h as described in *Section 5.2.1.2*, *Direct co-culture of hepatocytes with MSCs*.

The culture media were collected 24 h after culture. Culture supernatants were collected into sterile 1.5-mL centrifuge tubes and cryopreserved at −80°C for further assays. Cell cultures were rinsed with one-wash PBS and cryopreserved at −80°C for further experiments. All experiments were performed in duplicate and repeated in triplicate independently.

5.2.3 Hepatocyte apoptosis and total death assays

CCK18 assay was performed as described in ***Section 2.9*, *Caspase-cleaved CK18 Assay*** to determine caspase-mediated hepatocyte apoptosis. CK18 assay was performed as described in ***Section 2.10***, ***CK18 Assay*** to determine total hepatocyte death. The ratio of CCK18 to CK18 was also produced to determine the death mode of hepatocytes *in vitro*; a ratio of over 0.40 indicated that hepatocytes underwent apoptosis mainly, and necrosis *vice versa*. All experiments were performed in duplicate and repeated in triplicate independently.

5.2.4 Two-step, semi-quantitative, real-time polymerase-chained reaction assay

5.2.4.1 Total RNA extraction

The Direct-zol™ RNA MiniPrep kit (Zymo Research Corporation, Irvine, CA, USA) was used to isolate and purify RNA samples. Fresh cell cultures in 6-well plate were lysed by adding the TRIzol® reagent (Life Technologies Ltd., Paisley, UK), 1 mL per well. Cell sample homogenates were centrifuged at 12,000 × *g* for 1 min to remove any particulates and transferred into RNase-free Eppendorf tubes. One volume of absolute ethanol, molecular biology grade (Sigma-Aldrich, St Louis, MO, USA), was added into one volume of sample homogenate (1:1). The mixture was loaded into a Zymo-Spin™ IIC Column[2] and centrifuged in a collection tube at 12,000 × *g* for 1 min. The flow-through was discarded, and the Zymo-Spin™ column was transferred into a new collection tube. The concentrated (5 ×) RNA wash buffer was diluted with absolute ethanol and loaded into the Zymo-Spin™ column. The column was centrifuged 12,000 × *g* for 1 min, and the flow-through was discarded. The in-column DNase I digestion was performed using the DNase I cocktail (Promega, Madison, WI, USA) prepared as below (Table 5.1).

Table 5.1 In-column DNase I digestion cocktail recipe

Constituents	Volume per column (μL)
Lyophilised DNase I (1 U/μL)	5
10× RNase-free DNase I reaction buffer	8
DNase/RNase-free water	3
RNA wash buffer	64
Total	80

The cocktail was well mixed in an RNase-free Eppendorf tube by gentle inversion. The cocktail was directly loaded into the column matrix, 80 μL per column, and incubated on a heat block at 37°C for 15 min. The column was centrifuged at 12,000 × *g* for 30 sec. The Direct-zol™ RNA PreWash (5 ×, diluted in absolute ethanol) was loaded onto the column, 400 μL per column. The column was centrifuged at 12,000 × *g* for 1 min, and the flow-through was discarded. The step of prewash was repeated once. The RNA wash buffer was loaded onto the column, 700 μL per column, and the column was centrifuged at 12,000 × *g* for 1 min. The flow-through was discarded, and the column was centrifuged for additional 2 min. The column was transferred to an RNase-free Eppendorf tube. DNase/RNase-free water, 25 μL per column, was directly loaded into the column matrix, and the column was centrifuged at the maximum speed for 1 min. Additional 25-μL DNase/RNase-free water was added to further elute RNA sample. The quantity and purity of the eluted RNA sample were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA samples were reconstituted, at 1 μg for mono- or co-cultured hepatocytes and 0.5 per μg for mono-cultured MSCs per 26-μL final stock solution, in 0.2-mL PCR-clean Eppendorf tubes. An RNA sample with a ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) above 2.0 was determined to be pure RNA (Okamoto and Okabe, Int J Mol Med, 2000). The reconstituted stock solutions were stored at -20°C for further experiments.

5.2.4.2 cDNA synthesis

RNA samples were thawed on ice and reversely transcribed into complement DNA (cDNA) using the Omniscript® Reverse Transcriptase kit (Qiagen, West Sussex, UK). The reverse transcription (RT) cocktail was prepared using the following recipe (Table 5.2).

Table 5.2 Reverse transcription cocktail recipe

Constituents	Volume per sample (μL)
10× RT buffer	4
dNTP mix	4
Oligo dT primer	2
RNase out	0.5
Reverse transcriptase	2
DNase/RNase-free water	1.5
Total	14

RNA samples were preheated at 65°C using a PCR thermal cycler (Thermo Scientific, Wilmington, DE, USA) for 5min, and snap chilled on ice. The RT cocktail, 14 µL per vial, was added into the RNA sample, and the mixture was incubated at 37°C using the PCR thermal cycler for 60 min. The resultant cDNA samples were stored at -20°C for further experiments.

5.2.4.3 Semi-quantitative, real-time polymerase-chained reaction assay (qRT-PCR)

cDNA samples were thawed on ice and amplified using the TaqMan® PreAmp master mix kit (Applied Biosystems, Foster City, CA, USA). The qPCR Master Mix was prepared using the following recipe (Table 5.3).

Table 5.3 qPCR Master Mix recipe

Constituents	Volume per column (µL)
TaqMan® gene expression MasterMix	10
Primers	1
DNase/RNase-free water	7
Total	18

The qPCR Master Mix was added into a chilled MicroAmp® fast optical 96-well reaction plate Applied Biosystems, Foster City, CA, USA), 18 µL per well. The following proprietary primers (Table 5.4) for human gene expression assays were used and synthesised by Invitrogen (Paisley, UK). cDNA samples were added to the assigned wells, 2 µL per well, and well mixed by gentle pipetting.

Table 5.4 qPCR primer identification

Gene Symbol	Assay ID	Dye Label
B2M (reference gene)	Hs00984230_m1	VIC
CASP3	Hs00234385_m1	FAM
CASP8	Hs01018151_m1	FAM
CASP9	Hs00154261_m1	FAM
CASP14	Hs00201637_m1	FAM
BAX	Hs00180269_m1	FAM
BCL-2	Hs00236808_s1	FAM
BID	Hs00609632_m1	FAM
BLK	Hs00176441_m1	FAM

Semi qRT-PCR assay was performed using the ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: uracil-DNA glycosylase was activated at 50°C for 2 min; AmpliTaq® gold enzyme was activated at 50°C for 2 min; and cDNA template was denatured at 95°C for 15 sec and annealed/extended at 60°C for 1 min, for a total of 40 cycles. Cycle threshold (Ct) was produced with an automatic threshold using the Sequence Detection Software version 1.2.3 with 7000 System SDS Software RQ Study Application (Applied Biosystems, Foster City, CA, USA).

The qPCR results were analysed using the semi-quantitative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, Methods, 2001) with the following formulae [$\Delta\Delta Ct = \Delta Ct_{(treated)} - \Delta Ct_{(control)}$, where $\Delta Ct_{(treated/control)} = Ct_{(target\ gene)} - Ct_{(reference\ gene)}$], and expressed as mRNA expression level (fold) relative to the control (mono-cultured hepatocytes). A $2^{-\Delta\Delta Ct}$ value higher than 1 fold indicates upregulated expression of the target gene, and *vice versa*. All experiments were performed in duplicate and repeated in triplicate independently.

5.3 Results

5.3.1 Contribution of TNF-α and TGF-β to MSCs co-culture and HPc-induced potentiation

5.3.1.1 TNF-α secretion and neutralisation

Mono-cultured hepatocytes secreted a high level of TNF-α, while NPc-, HPc-, and NAC-pretreated HPc-MSCs secreted no detectable (below the lower assay limit) TNF-α (Figure 5.2A). Direct co-culture with NPc-MSCs significantly decreased hepatocyte secretion of TNF-α as compared to hepatocyte mono-culture, while indirect co-culture decreased TNF-α secretion to a significantly lesser extent (mono- *vs.* direct co- *vs.* indirect co-culture, 154.5 ± 12.6 *vs.*10.6 ± 1.3 *vs.* 134.1 ± 12.6 pg/mL, P <0.01). Co-culture with HPc-MSCs significantly further reduced hepatocyte secretion of TNF-α as compared to that with NPc-MSCs, while co-culture with NAC-pretreated HPc-MSCs significantly increased TNF-α secretion (NPc- *vs.* HPc- *vs.* NAC-treated HPc-MSCs co-culture, 10.6 ± 1.3 *vs.* undetectable *vs.* 10.3 ± 1.6 pg/mL, P <0.01).

TNF-α neutralisation even at the lowest dose (1×, 10 μg/mL) significantly inhibited CCK18 release from mono-cultured hepatocytes (0× *vs.* 1×, 64.6 ± 6.4 *vs.* 40.4 ± 6.2 U/10^6 hepatocytes, P <0.01), while TNF-α neutralisation at higher doses did not significantly further inhibit apoptosis of mono-cultured hepatocytes (Figure 5.2B). Similarly, TNF-α neutralisation at 2.5× significantly suppressed total death of hepatocytes, although that at 1× showed no significant effect (0× *vs.* 1× *vs.* 2.5×, 114.5 ± 7.2 *vs.* 105.9 ± 8.1 *vs.* 99.2 ± 7.3 U/10^6 hepatocytes, P <0.01; Figure 5.2C). TNF-α neutralisation switched the death mode of mono-cultured hepatocytes from apoptosis to necrosis (0× *vs.* 1× *vs.* 2.5×, 56.4% ± 4.5% *vs.* 38.1% ± 5.9% *vs.* 38.7% ± 4.8%, P <0.01; Figure 5.2D).

5.3.1.2 TGF-β1 secretion and neutralisation

Mono-cultured hepatocytes and MSCs secreted high levels of TGF-β1. HPc significantly increased MSCs secretion of TGF-β1, while NAC pretreatment significantly decreased TGF-β1 secretion (NPc *vs.* HPc *vs.* HPc+NAC, 172.5 ± 15.1 *vs.* 784.0 ± 35.0 *vs.* 596.9 ± 46.0 pg/mL, P <0.01; Figure 5.3A). Direct co-culture with NPc-MSCs significantly increased TGF-β1 secretion as compared to hepatocyte or MSCs mono-culture, while indirect co-culture increased TGF-β1 secretion to a significantly lesser extent (mono- *vs.* direct co- *vs.* indirect co-culture, 369.5 ± 19.6 *vs.* 871.0± 44.6 *vs.* 370.3 ± 22.4 pg/mL, P <0.01). Co-culture with HPc-MSCs further increased TGF-β1 secretion as compared to that with NPc-MSCs, while co-culture with NAC-pretreated HPc-MSCs significantly decreased TGF-β1 secretion (NPc- *vs.* HPc- *vs.* NAC-treated HPc-MSCs co-culture, 871.0± 44.6 *vs.* 1,370.8 ± 101.0 *vs.* 1,017.7 ± 64.1 pg/mL, P <0.01).

TGF-β1 neutralisation starting from 5× (1× = 1 μg/mL) significantly diminished antiapoptotic effect of NPc- and HPc-MSCs co-culture (0× *vs.* 5×, NPc: 35.2 ± 2.4 *vs.* 45.0 ± 1.8 U/10^6 hepatocytes, P <0.01; HPc: 32.0 ± 2.2 *vs.* 40.4 ± 2.8 U/10^6 hepatocytes, P <0.01; Figure 5.3B). Similarly, TGF-β1 neutralisation at 5× significantly diminished prosurvival effect of NPc- and HPc-

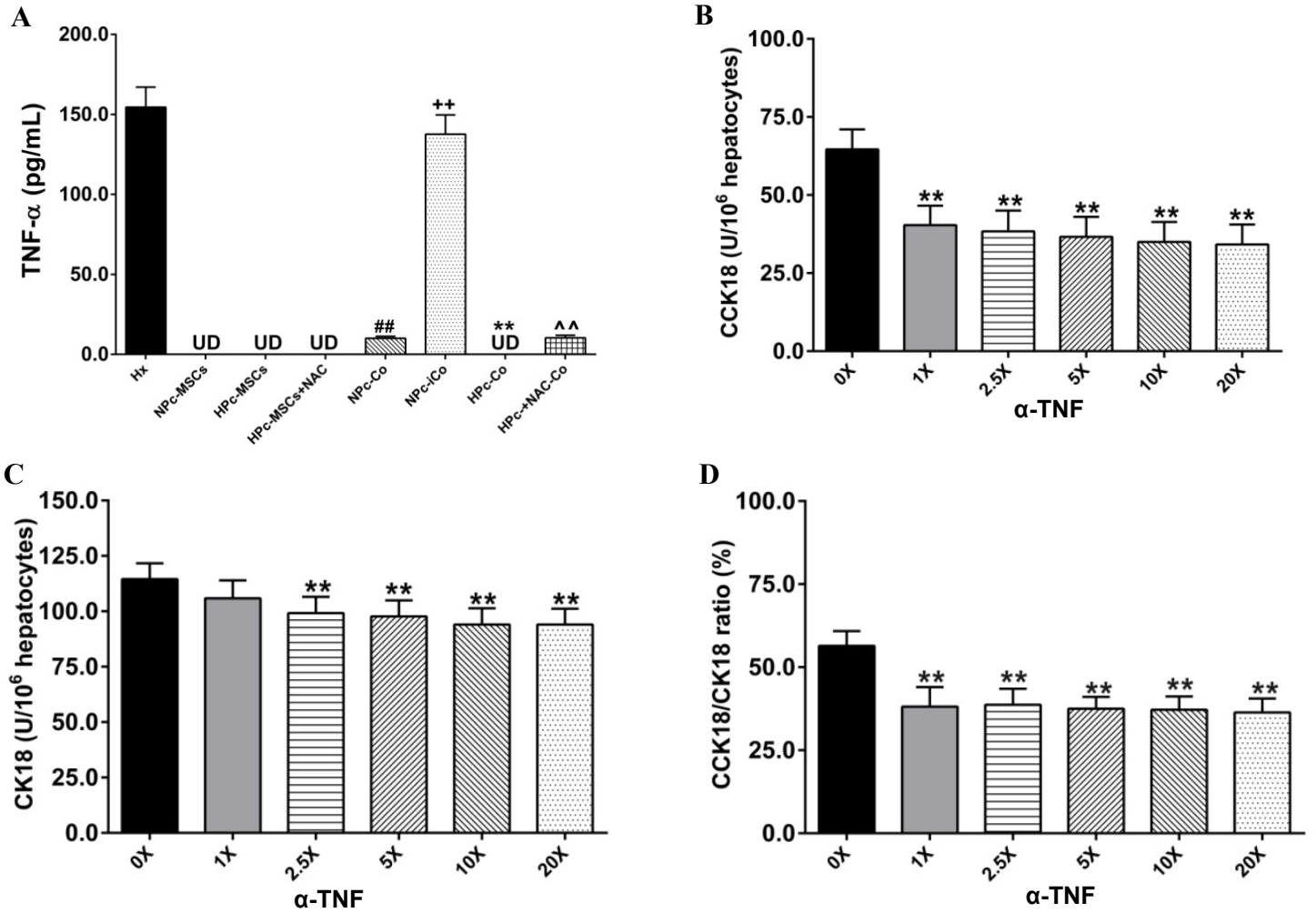

Figure 5.2 TNF-α ELISA and neutralisation experiments. TNF-α secretion of mono-/co-cultured hepatocytes (A); effects of autocrine TNF-α neutralisation on apoptosis (B), total death (C), and death mode (D) of mono-cultured hepatocytes. All data were expressed as mean ± SD; $^{**}P$ <0.01 *versus* control mono-/co-culture; $^{\wedge\wedge}P$ <0.01 *versus* non-NAC treated; $^{\#\#}P$ <0.01 *versus* control mono-culture; ^{++}P <0.01 *versus* direct co-culture. Hx, mono-cultured hepatocytes; UD, undetectable; iCo, indirect co-culture; α-TNF, anti-TNF.

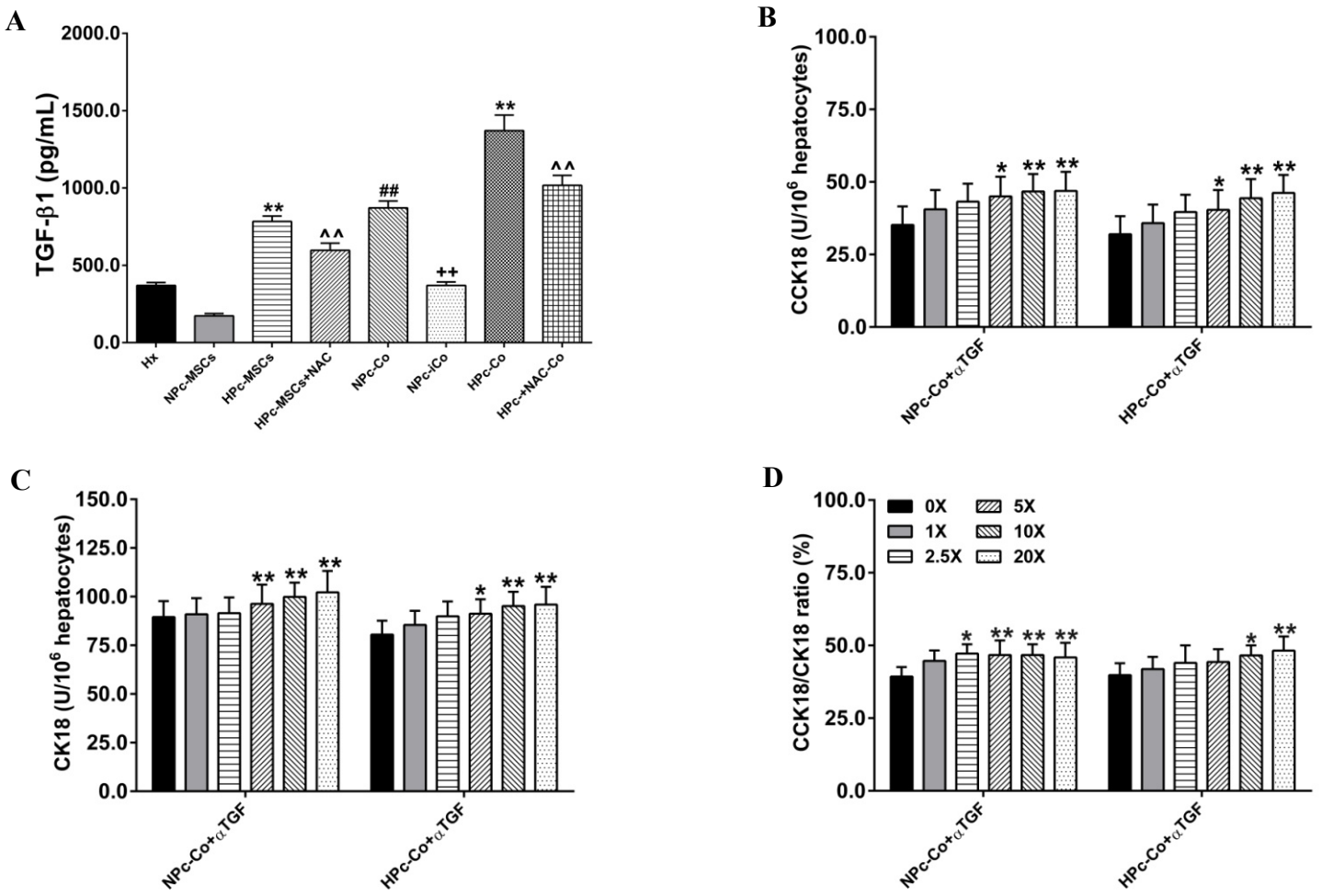

Figure 5.3 **TGF-β1 ELISA and neutralisation experiments.** TGF-β1 secretion of mono-/co-cultured hepatocytes and NPc-/HPc-MSCs (A); effects of MSCs TGF-β1 neutralisation on apoptosis (B), total death (C), and death mode (D) of co-cultured hepatocytes. All data were expressed as mean ± SD; **P* <0.05 and ***P* <0.01 *versus* control mono- or co-culture; ^^*P* <0.01 *versus* non-NAC treated; $^{\#\#}P$ <0.01 *versus* control mono-culture; ^{++}P <0.01 *versus* direct co-culture. Hx, mono-cultured hepatocytes; UD, undetectable; iCo, indirect co-culture; α-TGF, anti-TGF.

MSCs co-culture (0× *vs.* 5×, NPc: 89.5 ± 8.2 *vs.* 96.3 ± 9.9 U/10^6 hepatocytes, P <0.05; HPc: 80.5 ± 7.1 *vs.* 91.2 ± 7.4 U/10^6 hepatocytes, P <0.05; Figure 5.3C). TGF-β1 neutralisation resulted in a necrosis-to-apoptosis switch in death mode of hepatocytes co-cultured with NPc-MSCs starting from 1× (0× *vs.* 1×, 39.3% ± 3.3% *vs.* 44.7% ± 3.6%, P <0.05) and with HPc-MSCs from 10× (0× *vs.* 10×, 39.8% ± 4.1% *vs.* 46.6% ± 3.4%, P <0.05; Figure 5.3D).

5.3.2 Contribution of cellular and extracellular collagen

5.3.2.1 Cellular and extracellular collagen content

Hepatocytes expressed some cellular rather than extracellular collagen in mono- (data not shown) and co-culture (Figure 5.4A), while MSCs secreted massive cellular and extracellular collagen in both mono- (Figure 5.4B) and co-culture (Figure 5.4A).

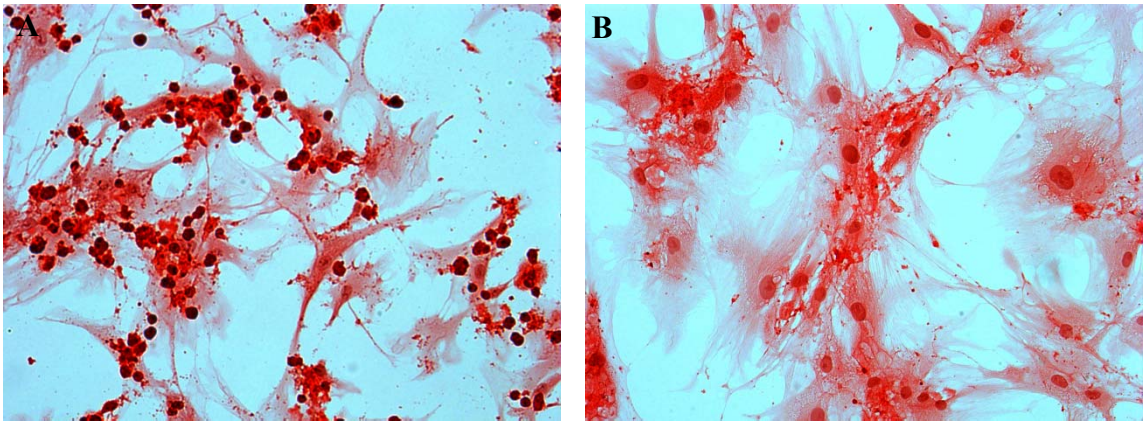

Figure 5.4 PSR collagen staining (200×) of hepatocytes in co-culture with MSCs (A) and MSCs in mono-culture (B). Intracellular collagen was found in hepatocytes and MSCs, and extracellular collagen was mainly located in proximity to MSCs.

HPc significantly increased MSCs expression of cellular collagen, while NAC pretreatment significantly decreased cellular collagen expression (NPc *vs.* HPc *vs.* HPc+NAC, 0.19 ± 0.04 *vs.* 0.31 ± 0.06 *vs.* 0.24 ± 0.04 OD units, P <0.01; Figure 5.5A). Direct co-culture with NPc-MSCs significantly increased cellular collagen expression as compared to hepatocyte or MSCs mono-culture, while indirect co-culture increased cellular collagen expression to a significantly lesser extent (mono- *vs.* direct co- *vs.* indirect co-culture, 0.07 ± 0.01 *vs.* 0.30 ± 0.06 *vs.* 0.22 ± 0.03 OD units, P <0.01). Co-culture with HPc-MSCs further increased cellular collagen expression as compared to that with NPc-MSCs, while co-culture with NAC-pretreated HPc-MSCs significantly decreased cellular collagen expression (NPc- *vs.* HPc- *vs.* NAC-treated HPc-MSCs co-culture, 0.30 ± 0.06 *vs.* 0.55 ± 0.04 *vs.* 0.33 ± 0.04 OD units, P <0.01).

Mono-cultured hepatocytes secreted minimal extracellular collagen (below the lower assay limit), while MSCs deposited extracellular collagen (Figure 5.5B). HPc significantly increased MSCs deposition of extracellular collagen, while NAC pretreatment significantly decreased

extracellular collagen deposition (NPc *vs.* HPc *vs.* HPc+NAC, 0.39 ± 0.04 *vs.* 0.56 ± 0.06 *vs.* 0.40 ± 0.02 mg/10^6 cells, P <0.01). Direct co-culture with NPc-MSCs significantly increased extracellular collagen deposition as compared to hepatocyte or MSCs mono-culture, while indirect co-culture increased extracellular collagen deposition to a significantly lesser extent (mono- *vs.* direct co- *vs.* indirect co-culture, 0.39 ± 0.04 *vs.* 2.89 ± 0.26 *vs.* 0.41 ± 0.02 mg/10^6 cells, P <0.01). Co-culture with HPc-MSCs further increased cellular collagen expression as compared to that with NPc-MSCs, while co-culture with NAC-pretreated HPc-MSCs significantly decreased cellular collagen expression (NPc- *vs.* HPc- *vs.* NAC-treated HPc-MSCs co-culture, 2.89 ± 0.26 *vs.* 5.97 ± 0.53 *vs.* 3.30 ± 0.30 mg/10^6 cells, P <0.01).

5.3.2.2 Effects of MaIBA on NPc- and HPc-MSCs co-culture

MaIBA at a dose range from 0.1 to 20 mM had no toxic effect on MSCs with respect to mitochondrial dehydrogenase activity and cellular attachment (Table 5.5). MaIBA pretreatment at 5, 10, and 20 mM significantly inhibited extracellular collagen deposition of NPc- and HPc-MSCs (0 mM *vs.* 5 mM *vs.* 10 mM *vs.* 20 mM, NPc: 0.39 ± 0.04 *vs.* 0.33 ± 0.03 *vs.* 0.32 ± 0.03 *vs.* 0.30 ± 0.02 mg/10^6 cells, P <0.01, Figure 5.5C; HPc: 0.56 ± 0.06 *vs.* 0.45 ± 0.05 *vs.* 0.44 ± 0.04 *vs.* 0.42 ± 0.04 mg/10^6 cells, P <0.01; Figure 5.5D); therefore, 5-mM MaIBA was used for further collagen inhibition experiment.

Table 5.5 Cytotoxic effect to MaIBA on MSCs

MaIBA (mM)	MTT (OD unit)	SRB (OD unit)
0	0.35 ± 0.03	0.53 ± 0.05
0.1	0.33 ± 0.02	0.51 ± 0.04
0.5	0.33 ± 0.03	0.51 ± 0.06
1	0.33 ± 0.02	0.50 ± 0.03
2.5	0.32 ± 0.03	0.53 ± 0.03
5	0.32 ± 0.03	0.49 ± 0.03
10	0.31 ± 0.03	0.51 ± 0.06
20	0.33 ± 0.03	0.50 ± 0.03

Pretreatment of 5-mM MaIBA significantly inhibited extracellular collagen deposition of hepatocytes co-cultured with NPc- and HPc-MSCs (0 mM *vs.* 5 mM, NPc: 2.89 ± 0.26 *vs.* 2.03 ± 0.28 mg/10^6 cells, P <0.01; HPc: 5.97 ± 0.23 *vs.* 4.47 ± 0.29 mg/10^6 cells, P <0.01; Figure 5.6A). MaIBA pretreatment significantly diminished antiapoptotic effect of MSCs co-culture (0 mM *vs.* 5 mM, 18.5 ± 2.4 *vs.* 25.5 ± 2.5 U/10^6 hepatocytes, P <0.01) and potentiative effect of HPc induction (14.5 ± 2.3 *vs.* 20.8 ± 2.1 U/10^6 hepatocytes, P <0.01; Figure 5.6B). However, MaIBA pretreatment had no significant prosurvival effect of MSCs co-culture and potentiative effect of HPc induction (Figure 5.6C). MaIBA pretreatment resulted in a necrosis-to-apoptosis switch in hepatocytes co-cultured with NPc-MSCs (30.2% ± 3.2% *vs.* 38.9% ± 4.5%, P <0.01) and with HPc-MSCs (28.1% ± 3.0% *vs.* 35.4% ± 4.5%, P <0.01; Figure 5.6D).

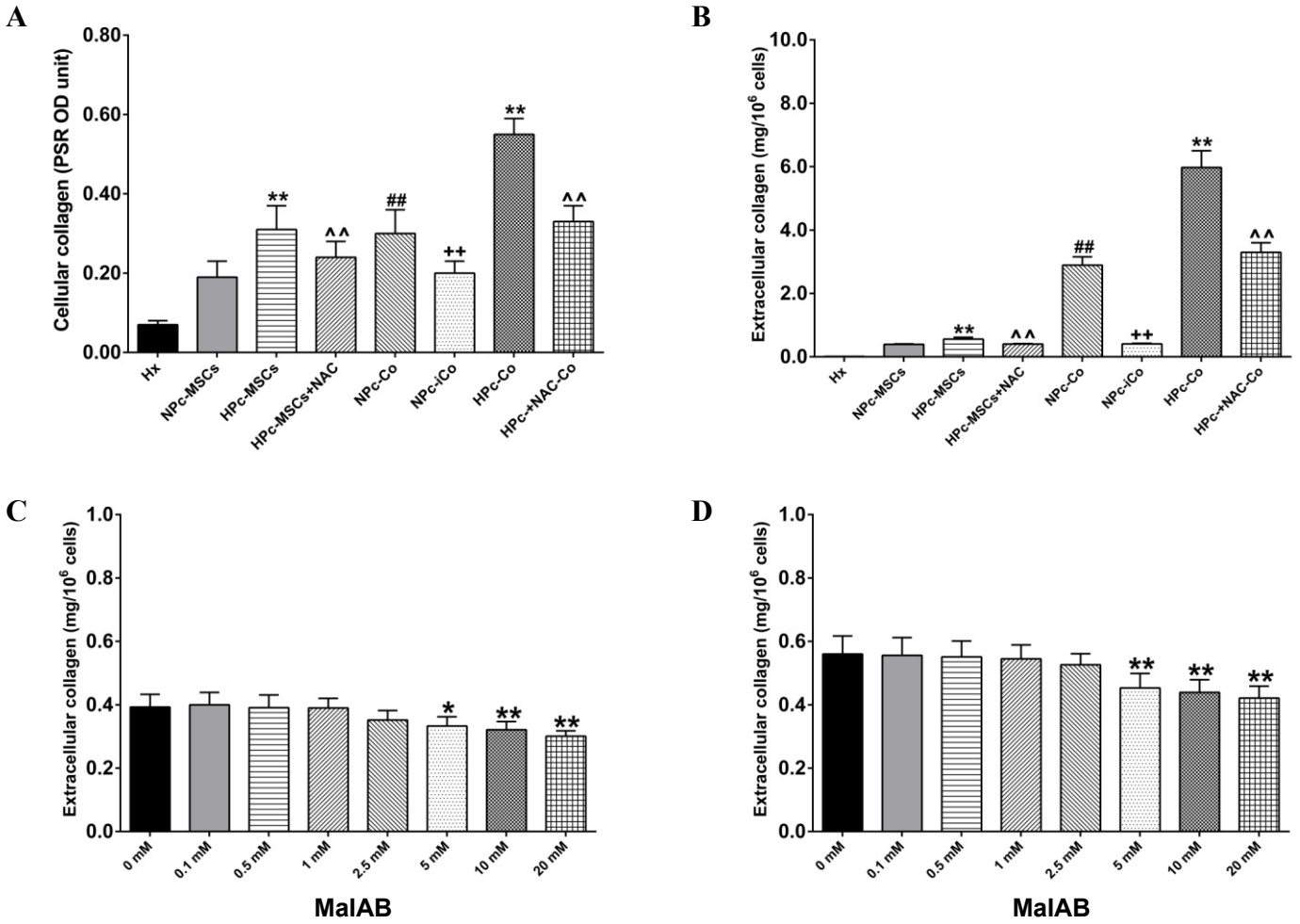

Figure 5.5 Cellular and extracellular collagen assays and collagen inhibition experiment. Cellular (A) and extracellular collagen (B) content of mono-/co-cultured hepatocytes and NPc-/HPc-MSCs; inhibitory effect of 0- to 20-mM MaIBA on extracellular collagen deposit of NPc- (C) and HPc-MSCs (D). All data were expressed as mean ± SD; **P* <0.05 and ***P* <0.01 *versus* control mono- or co-culture; ^^*P* <0.01 *versus* non-NAC treated; $^{\#\#}P$ <0.01 *versus* control mono-culture; ^{++}P <0.01 *versus* direct co-culture. Hx, mono-cultured hepatocytes; UD, undetectable; Co, co-culture; iCo, indirect co-culture.

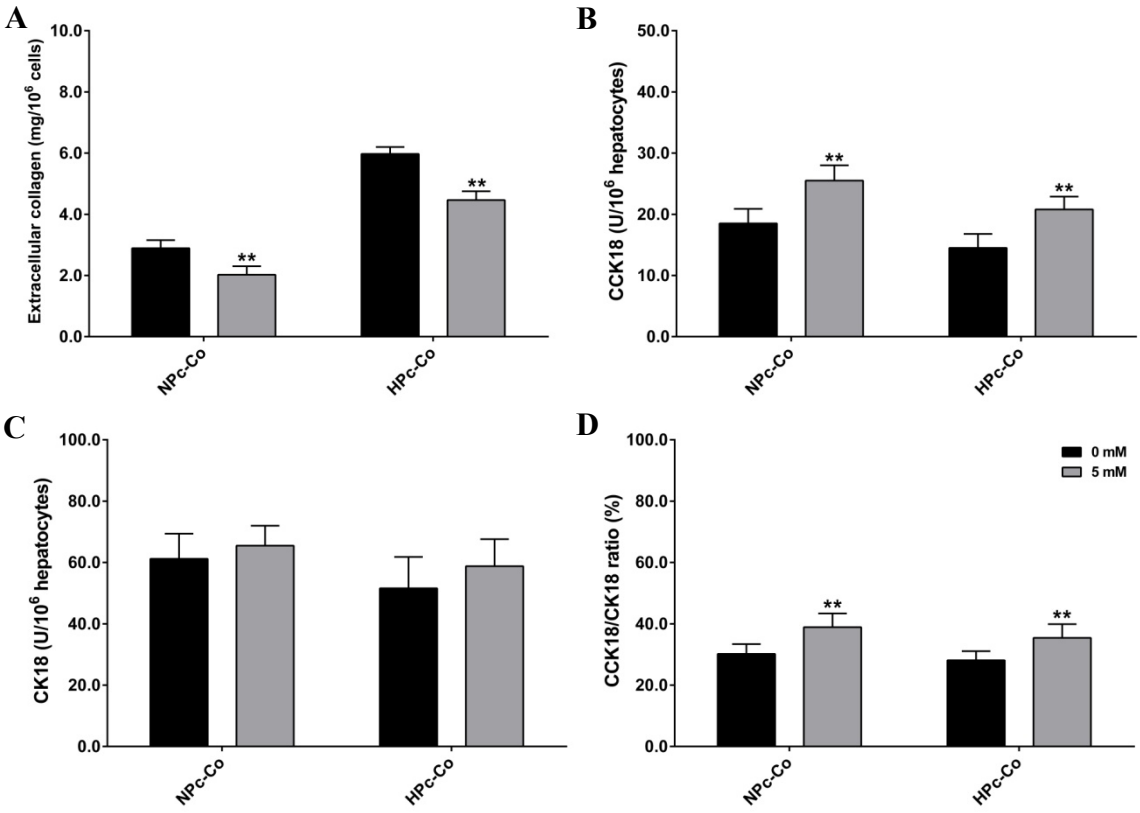

Figure 5.6 Effects of 5-mM MaIBA pretreatment on extracellular collagen deposit (A), cellular apoptosis (B), total death (C), and death mode (D) of hepatocytes co-cultured with NPc-*versus* HPc-MSCs. All data were expressed as mean ± SD; ***P* <0.01 *versus* NPc-Co. Co, co-culture.

5.3.3 Pro- and antiapoptosis-associated gene expressions

5.3.3.1 CASP9

Expression of *CASP3*, *CASP8*, and *CASP14* mRNA was undetectable in mono- or co-cultured hepatocytes. MSCs co-culture significantly downregulated expression of *CASP9* mRNA (0.72 ± 0.07 fold, *P* <0.01), while indirect co-culture slightly downregulated *CASP9* mRNA expression (0.90 ± 0.07 fold, *P* >0.0; Figure 5.7A). HPc further downregulated *CASP9* mRNA expression in MSCs co-culture (0.55 ± 0.05 fold, *P* <0.01), while NAC pretreatment diminished HPc-induced further downregulation (0.71 ± 0.07 fold, *P* <0.01). Additionally, staurosporine pretreatment significantly upregulated expression of *CASP9* mRNA in mono-cultured hepatocytes (1.44 ± 0.12 folds, *P* <0.01), while MSCs co-culture significantly decreased staurosporine-induced upregulation of *CASP9* mRNA expression (0.90 ± 0.07 fold, *P* <0.01).

5.3.3.2 BAX, BCL-2, BAX/BCL-2 ratio, and BID

MSCs co-culture significantly downregulated expression of *BAX* mRNA (0.39 ± 0.11 fold, *P* <0.01), while indirect co-culture slightly downregulated *BAX* mRNA expression (0.83 ± 0.17 fold, *P* >0.05; Figure 5.7B). HPc further downregulated *BAX* mRNA expression in MSCs co-culture (0.12 ± 0.03 fold, *P* <0.01), while NAC pretreatment diminished HPc-induced further downregulation (0.26 ± 0.10 fold, *P* <0.01). Additionally, staurosporine pretreatment significantly upregulated

expression of *BAX* mRNA in mono-cultured hepatocytes (1.37 ± 0.24 folds, P <0.01), while MSCs co-culture significantly decreased staurosporine-induced upregulation of *BAX* mRNA expression (0.65 ± 0.17 fold, P <0.01).

MSCs co-culture significantly upregulated expression of *BCL-2* mRNA (1.28 ± 0.17 fold, P <0.01), while indirect co-culture slightly downregulated *BCL-2* mRNA expression (0.95 ± 0.10 fold, P >0.05; Figure 5.7C). HPc further upregulated *BCL-2* mRNA expression in MSCs co-culture (1.86 ± 0.26 folds, P <0.01), while NAC pretreatment diminished HPc-induced further upregulation (1.34 ± 0.09 folds, P <0.01). Additionally, staurosporine pretreatment significantly downregulated expression of *BCL-2* mRNA in mono-cultured hepatocytes (0.58 ± 0.17 fold, P <0.01), while MSCs co-culture significantly increased staurosporine-induced downregulation of *BCL-2* mRNA expression (0.86 ± 0.10 fold, P <0.01).

MSCs co-culture significantly decreased *BAX*/*BCL-2* ratio (0.30 ± 0.07 fold, P <0.01), while indirect co-culture slightly decreased *BAX*/*BCL-2* ratio (0.88 ± 0.12 fold, P >0.05; Figure 5.7D). HPc further decreased *BAX*/*BCL-2* ratio in MSCs co-culture (0.06 ± 0.01 fold, P <0.01), while NAC pretreatment diminished HPc-induced further decrease (0.20 ± 0.07 fold, P <0.01). Additionally, staurosporine pretreatment significantly increased *BAX*/*BCL-2* ratio in mono-cultured hepatocytes (2.36 ± 0.22 folds, P <0.01), while MSCs co-culture significantly decreased staurosporine-induced *BAX*/*BCL-2* ratio increase (0.76 ± 0.12 fold, P <0.01).

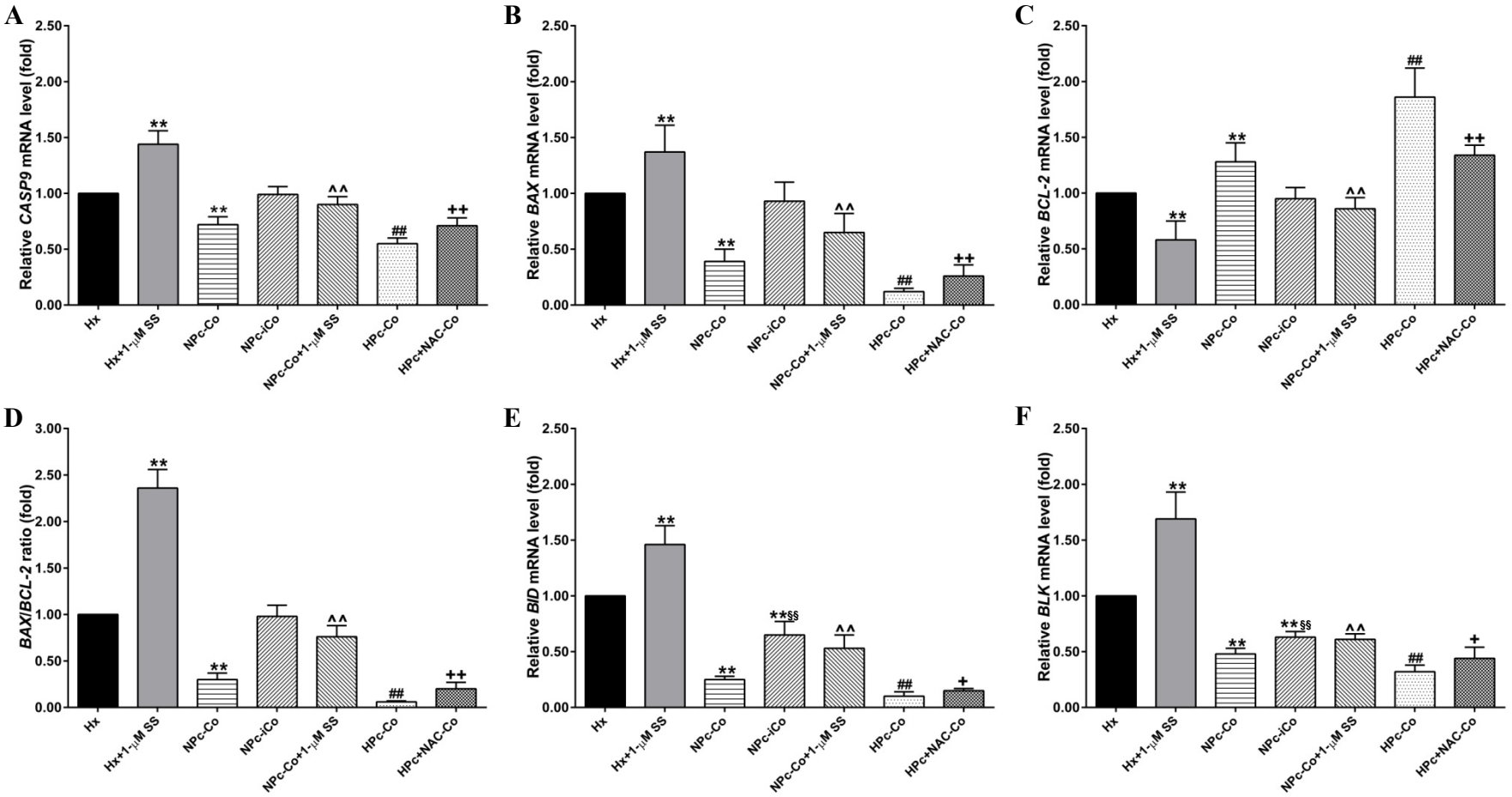

Figure 5.7 Relative mRNA expression levels of pro- and antiapoptosis-associated genes in mono- and co-cultured hepatocytes: *CASP9* (A), *BAX* (B), *BCL-2* (C), *BAX*/*BCL-2* ratio (D), *BID* (E), and *BLK* (F). All data were expressed as mean ± SD; $^{**}P$ <0.01 *versus* control hepatocyte mono-culture; $^{\S\S}P$ <0.01 *versus* direct co-culture; $^{\wedge\wedge}P$ <0.01 *versus* SS-treated, mono-cultured hepatocytes; $^{\#\#}P$ <0.01 *versus* NPc co-culture; ^{+}P < 0.05 and ^{++}P <0.01 *versus* non-NAC-treated HPc co-culture. Hx, mono-cultured hepatocytes; SS, staurosporine; Co, co-culture; iCo, indirect co-culture.

MSCs co-culture significantly downregulated expression of *BID* mRNA (0.25 ± 0.13 fold, P <0.01), and indirect co-culture also significantly downregulated *BID* mRNA expression to a significantly lesser extent (0.65 ± 0.12 fold, P <0.01; Figure 5.7E). HPc further downregulated *BID* mRNA expression in MSCs co-culture (0.10 ± 0.04 fold, P <0.01), while NAC pretreatment diminished HPc-induced further downregulation (0.15 ± 0.02 fold, P <0.01). Additionally, staurosporine pretreatment significantly upregulated expression of *BID* mRNA in mono-cultured hepatocytes (1.46 ± 0.17 folds, P <0.01), while MSCs co-culture significantly decreased staurosporine-induced upregulation of *BID* mRNA expression (0.53 ± 0.12 fold, P <0.01).

5.3.3.3 BLK

MSCs co-culture significantly downregulated expression of *BLK* mRNA (0.48 ± 0.05 fold, P <0.01), and indirect co-culture also significantly downregulated *BLK* mRNA expression to a significantly lesser extent (0.63 ± 0.05 fold, P <0.01; Figure 5.7F). HPc further downregulated *BLK* mRNA expression in MSCs co-culture (0.32 ± 0.06 fold, P <0.01), while NAC pretreatment diminished HPc-induced further downregulation (0.44 ± 0.12 fold, P <0.01). Additionally, staurosporine pretreatment significantly upregulated expression of *BLK* mRNA in mono-cultured hepatocytes (1.69 ± 0.24 folds, P <0.01), while MSCs co-culture significantly decreased staurosporine-induced upregulation of *BLK* mRNA expression (0.61 ± 0.05 fold, P <0.01).

5.3.3.4 Summary of gene expression assays

Direct MSCs co-culture significantly downregulated expression of proapoptotic *CASP9*, *BAX*, and *BID* mRNA and significantly upregulated expression of antiapoptotic *BCL-2* mRNA. In contrast, indirect MSCs co-culture significantly downregulated expression of *BID* and *BLK* mRNA to a significantly lesser extent only. HPc further significantly downregulated expression of *CASP9*, *BAX*, *BID*, and *BLK* mRNA and significantly upregulated expression of *BCL-2* mRNA in MSCs co-culture; however, NAC pretreatment diminished HPc-induced down- and upregulation of the respective genes. Additionally, staurosporine pretreatment culture significantly upregulated expression of *CASP9*, *BAX*, *BID*, and *BLK* mRNA and significantly downregulated expression of *BCL-2* mRNA, while MSCs co-culture diminished staurosporine-induced down- and upregulation of the respective genes.

5.4 Discussion

5.4.1 MSCs inhibit autocrine TNF-α activity of co-cultured hepatocytes

TNF-α is a pleiotropic cytokine chiefly secreted by activated immune cells, and plays a central role in inflammation and apoptosis. TNF-α is known to mediate LPS-induced hepatocyte apoptosis, manifesting as DNA fragmentation and cytoplasmic translocation of alanine aminotransferase, which can be antagonised by pretreatment with IL-1β (Leist *et al.*, 1995). TNF-α induced hepatocyte apoptosis depends on caspase signalling pathway, including activation of caspases-2 (Guicciardi *et al.*, 2005), -8 (Imao *et al.*, 2006), and -9 (Imao *et al.*, 2006). Hepatocytes secrete a high level of TNF-α (600 pg/mL) within 4 h if challenged with LPS (Saad *et al.*, 1995). However, TNF-α inhibitors suppress hepatocyte proliferation in response to mitogen (Kubo *et al.*, 1996), while stimulatory effect of TNF-α on hepatocytes may result from liver NPCs response to TNF-α (Shinozuka *et al.*, 1996). It was also reported that hepatocytes resisted TNF-α induced apoptosis by a mechanism dependent on pre-existing intracellular glutathione, a major effector reducing ROS (Xu *et al.*, 1998). This bidirectional regulation of TNF-α on epithelial cells may be modulated by other extrinsic and/or intrinsic factors, such as redox (Kim *et al.*, 2000), NF-κB (Nagaki *et al.*, 2000), EGFR (Argast *et al.*, 2004), and HGF (Grant-Tschudy and Wira, 2005).

The present work showed that mono-cultured hepatocytes secreted a baseline level of autocrine TNF-α (approximately 640 pg/10^6 hepatocytes/24 h), and TNF-α neutralisation significantly decreased spontaneous apoptosis and total death of mono-cultured hepatocytes. MSCs expressed no TNF-α and inhibited autocrine activity of TNF-α in co-cultured hepatocytes. This finding was contradictory to that reported by Gu *et al.* (2009[a]), in which TNF-α was not expressed in mono- or co-cultured hepatocytes. This inconsistency might derive from the differences in source of hepatocytes (human *versus* porcine) and co-culture protocol (layered *versus* mixed). Shi *et al.* (2011) reported that MSCs co-culture protected hepatocytes from apoptosis induced by exposure to acute-on-chronic liver failure serum containing a high level of TNF-α. It was also reported that TNF-α treatment significantly upregulated expression of VEGF, FGF2, HGF, and IGF-1 in human MSCs by an NF-κB-independent, JNK-independent mechanism (Crisostomo *et al.*, 2008). The present work demonstrated for the first time that trophic and protective effects of MSCs on co-cultured hepatocytes might result from inhibition of proapoptotic TNF-α in addition to expression of trophic factors by MSCs. However, this finding should be cautiously explained as MSCs co-culture CM containing a very low level of TNF-α could not improve liver-specific metabolism or protect mono-cultured hepatocytes from spontaneous apoptosis, suggesting the role of synergistic factors aside from TNF-α. The present work showed that inhibitory effect of MSCs co-culture on autocrine TNF-α activity of hepatocytes depended on MSC-to-hepatocyte contact as evidenced by the minimal effect of indirect non-contact co-culture. This inhibitory effect could also be potentiated by HPc in an intra-MSCs ROS activity dependent manner as NAC pretreatment diminished the potentiative effect of HPc induction.

5.4.2 Hepatotrophic effect of MSCs co-culture and potentiative effect of HPc induction depend on autocrine TGF-β activity of MSCs

TGF-β is a polypeptide secreted by multiple cell lines, including platelets, macrophages, fibroblasts, and mesenchymal stem cells, and involved in a large number of biological activities, such as cell growth, cell proliferation, cell differentiation, and apoptosis (Ng *et al.*, 2008). TGF-β plays a crucial role in regulating MSCs differentiation and promotes chondrogenic differentiation of human MSCs by upregulating expression of SOX9, type II collagen, and aggrecan (Miyanishi *et al.*, 2006). Synergistic treatment of IL-1β and TGF-β1 can also increase MSCs secretion of VEGF and improve cardioprotective effect of MSCs transplantation (Luo *et al.*, 2012). TGF-β is also believed to be a major trophic factor released from MSCs for the host cells. MSCs maintain phenotype and pluripotency of human ES cells through TGF-β and FGF receptor in response to bFGF (Montes *et al.*, 2009).

Generally, TGF-β has a negative effect on biological activities of hepatocytes. TGF-β inhibits hepatocyte DNA synthesis, proliferation, differentiation, and liver-specific metabolism. TGF-β is well known as a potent stimulant that induces EMT. TGF-β treatment *in vitro* can induce EMT in human foetal hepatocytes accompanied by upregulation of Snail signalling (mesenchymal cell marker) and downregulation of E-cadherin (epithelial cell marker) expression (Caja *et al.*, 2011). Abnormal TGF-β expression is believed to be a major etiology of liver fibrosis (Dooley *et al.*, 2008; Ciuclan *et al.*, 2010). However, TGF-β1 was reported to be required for functional enhancement in hepatocytes co-cultured with NIH/3T3 cells as this functional enhancement could be eliminated by TGF-β1 depletion and restored by TGF-β1 reconstitution (Chia *et al.*, 2005). This paradox might result from the complex interplays between soluble factors and MSCs/hepatocytes.

The present work showed that both hepatocytes and MSCs secreted a baseline level of TGF-β1, while co-culture of hepatocytes with MSCs exhibited an additive or synergistic effect with respect to TGF-β1 production. This additive or synergistic effect depended on MSC-to-hepatocyte contact as indirect non-contact co-culture only secreted a similar level of TGF-β1 to direct co-culture. This finding was consistent with the previous report (Chia *et al.*, 2005). HPc potentiated MSCs secretion of TGF-β1 in both mono- and co-culture by an intracellular ROS-dependent manner as evidenced by the reversal effect of NAC pretreatment. It has recently been reported that hypoxia increases MSCs secretion of TGF-β1 and promotes breast cancer cell progression, and the major hypoxia-regulated element is determined to be HIF-1 binding to the hypoxia response element of TGF-β1 promoter (Hung *et al.*, 2013). The present work also demonstrated that autocrine TGF-β1 activity of MSCs was required for the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction as evidenced by neutralisation of MSCs-derived TGF-β1. As MSCs co-culture CM containing high-level TGF-β1 had no significant hepatotrophic and antiapoptotic effect, autocrine TGF-β1 of NPc- and HPc-MSCs might not act on hepatocytes through ECM and/or cell-to-cell contact rather than a paracrine mechanism. TGF-β activation is reported to mediate the crosstalk between hepatocytes and the stromal niche in the setting of HCV infection (Benzoubir *et al.*, 2013).

5.4.3 Hepatotrophic effect of MSCs co-culture and potentiative effect of HPc induction depend on MSCs deposition of extracellular collagen

Collagen is the major component and the most abundant protein in ECM. The main biological function of collagen, especially extracellular form, is to support resident cells in the form of fibrillar protein. In physiological conditions, liver collagen is mainly produced by hepatic NPCs rather than hepatocytes, while pathological hepatocytes deposit collagen in response to profibrotic factors, such as TGF-β (Vadasz *et al.*, 2005). The present work confirmed that hepatocytes secreted little extracellular collagen; however, hepatocytes were reported to produce some collagenous components, such as collagen type I, II, III, and V (Diegelmann *et al.*, 1983). Collagen attachment is known to support hepatocyte with respect to metabolic function; as a result, monolayer collagen coating and modified collagen gel ‘‘sandwich’’ culture are widely used for hepatocyte culture and bioartificial liver system (Wang *et al.*, 2004). Maintenance effect of collagen was thought to result from persistent phosphorylation of HGF and EGF receptors (Engl *et al.*, 2004). Extracellular collagen also regulates the cell cycle of hepatocytes. Collagen facilitates the entry of hepatocytes into the S-phase by a cyclin D1-dependent mechanism (Hansen and Albrecht, 1999), and mediates aggregation of hepatocytes and intercellular contact (Moghe *et al.*, 1997).

Collagen regulates survival, proliferation, and differentiation of MSCs; conversely, MSCs deposit extracellular collagen during chondrogenesis and osteogenesis (Li *et al.*, 2011). The present work demonstrated that MSCs deposition of extracellular collagen could be enhanced by direct co-culture with hepatocytes, and extracellular collagen deposition contributed, at least partially, to trophic and antiapoptotic effects of MSCs on co-cultured hepatocytes as shown in the collagen inhibition experiments. This finding was consistent with the report by Gu *et al.* (2009[c]); extracellular collagen was mainly located around MSCs and knockdown of collagen type I/V expression in MSCs significantly reduced synthesis of albumin and urea in hepatocytes. This finding also suggested that enhanced MSCs deposition of extracellular collagen might result primarily from MSC-to-hepatocyte contact as indirect non-contact co-culture did not show any significant effect. The present work also showed that HPc significantly increased extracellular collagen expression in mono- and co-cultured MSCs in an intracellular ROS dependent manner. Low oxygen tension is known to promote osteochondrogenesis of MSCs. HPc significantly upregulated expression of collagen I, II, and X along with some other genes encoding ECM (Müller *et al.*, 2011). Hypoxia can also optimise cartilaginous matrix production of articular chondrocytes co-cultured with MSCs, thereby minimizing the requirement on harvest and expansion of primary chondrocytes (Meretoja *et al.*, 2013). It was noted that increased MSCs deposition of extracellular collagen was accompanied by enhanced autocrine activity of TGF-β, a potent stimulant of collagen synthesis (Rodríguez *et al.*, J Cell Biochem, 2000). Increased extracellular collagen deposit might result from autocrine TGF-β activation in MSCs.

5.4.4 Hepatotrophic effect of MSCs co-culture and potentiative effect of HPc induction result from downregulation of proapoptotic signalling and upregulation of antiapoptotic signalling

Apoptotic death of hepatocytes is finely modulated by the complex interplay between proapoptotic factors and antiapoptotic factors. Spontaneous hepatocyte apoptosis is thought to be a major cause of early cellular loss and metabolic deterioration. The well documented trophic effect of MSCs on co-cultured hepatocytes may result from suppression of hepatocyte apoptosis. The antiapoptotic effect of MSCs is supposed to derive from a combination of paracrine factors, ECM, and cell-to-cell interaction. The results in Chapter 3 showed that MSCs significantly inhibited spontaneous and chemically-induced apoptosis of co-cultured hepatocytes by nonparacrine mechanisms, and those in Chapter 4 further confirmed that HPc potentiated antiapoptotic effect of MSCs co-culture by an intracellular ROS dependent mechanism. The results of gene expression analyses in this chapter showed that downregulation of apoptosis-associated caspases and BAX/BCL-2 signalling pathways underlay the antiapoptotic effect of MSCs and potentiative effect of HPc induction. To the best of my knowledge, the present work demonstrated apoptosis-associated gene expression profiles of hepatocytes co-cultured with MSCs for the first time. It was also noted that indirect co-culture with MSCs only downregulated expression of two genes, BID and BLK. This finding might explain why paracrine factors of MSCs had limited trophic and antiapoptotic effect on co-cultured hepatocytes.

Caspases, also called cysteine-dependent aspartate-directed proteases, are a family of cysteine proteases that play an essential role in regulating cellular apoptosis, necrosis, and inflammatory response. Caspase signalling pathway functions to initiate and execute cellular apoptosis in a complex but well documented cascade manner, if activated by granzyme B for caspases-3 and -7, death receptors for caspases-8 and -10, and apoptosome for caspase-9. Caspase-3 plays a central role in the execution phase of apoptosis activated both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways, and interacts with caspases-8 and-9. LPS-induced hepatocyte apoptosis was thought to result from upregulation of caspase-3 expression in the presence of activated Kupffer cells (Hamada *et al.*, 1999). AT-MSCs CM was reported to protect PC12 cells from glutamate excitotoxicity-induced apoptosis by inhibiting caspase-3 activity through upregulation of XIAP (X-linked inhibitor of apoptosis protein) and PI3-K (phosphatidylinositide 3-kinases)/Akt activation (Lu *et al.*, 2011). Caspase-8 is the prototypical apoptosis initiator downstream of TNF superfamily death receptors, and activated caspase-8 can start the cascades of cellular apoptosis along with other effector caspases and proapoptotic Bcl-2 family members. ROS-induced activation of caspase-8 was reported to promote hepatocyte apoptosis and liver fibrosis in the setting of non-alcoholic steatohepatitis (Hatting *et al.*, 2013). Caspase-9 is the initiator of caspase-mediated apoptosis through the mitochondrial pathway; activation of JNK/SAPK (stress-activated protein kinase) signalling releases cytochrome from the mitochondrial into the cytoplasm and activates the apoptosome, which cleaves the pro-enzyme of caspase-9 into the active form (Kang and Chae, 2003). Caspase-14 is activated by caspases-8 and -9 and invovled in apoptosis execution and also

keratinocyte terminal differentiation (Walsh *et al.*, 2005). The role of caspase-14 remains unknown in hepatocyte apoptosis. The present work showed that MSCs co-culture and HPc significantly downregulated expression of caspase-9; however, no expression of caspases-3, -8, and -14 was detected. This finding suggested that antiapoptotic effect of MSCs co-culture and potentiative effect of HPc induction mainly acted on initiation of apoptosis, consistent with reduced caspase-9 initiated cleavage of hepatocyte CK18 into CCK18.

BCL-2 is a superfamily of regulator proteins, including Bax, Bcl-2, Bid, invovled in cellular survival and apoptosis. Bax (Bcl-2-associated X protein 4) was the first identified proapoptotic member of Bcl-2 protein family. Following induction of apoptosis, BAX interacts with the mitochondrial membrane and activate the voltage-dependent anion channel. The opening of the anion channel results in massive release of cytochrome c and other proapoptotic factors and further activates caspases. In contrast, Bcl-2 (B-cell lymphoma 2) is an antiapoptotic member, and prevents the activation of BAX signalled by the ‘‘death cue’’ along with Bcl-xL. The Bax/Bcl-2 balance is well known to regulate hepatocyte apoptosis (Hikita *et al.*, 2011). BID (BH3 interacting-domain death agonist) interacts with Bax and leads to Bax insertion into the outer mitochondrial membrane in response to apoptotic signals. Activation of the death receptor with Fas ligand leads to activation of caspase-3 and -8 as mediated by Bid-dependent mitochondrial release of Smac in the apoptotic event of hepatocytes (Li *et al.*, 2002). Bid is also upstream of caspase-2 activation in the setting of TNF-α triggered hepatocyte apoptosis (Guicciardi *et al.*, 2005). Park *et al.* (2010) reported that MSCs co-culture upregulated expression of Bcl-xL and Bcl-2 in islet cells. Autologous transplantation of AT-MSCs alleviated ischaemia/reperfusion-induced hepatocyte apoptosis by upregulating Bcl-2 (Sun *et al.*, 2012). The present work showed that co-culture with NPc-MSCs significantly decreased *BAX*/*BCL-2* ratio and *BID* expression in a cell-contact dependent manner, while that with HPc-MSCs exhibited a significantly greater effect by an intracellular ROS-dependent mechanism.

BLK (B lymphoid tyrosine kinase) is a member of SRC non-receptor tyrosine kinase subfamily involved in B-lymphocyte development, differentiation and signalling. BLK is mainly present in lymphatic tissues, pancreatic islets, and also highly expressed in colon epithelial cells (Seidelin and Nielsen, 2006). BLK protein enhances islet cell synthesis and secretion of insulin in response to glucose and upregulates expression of pancreatic β-cell transcription factors. The biological role of BLK in hepatocyte biology remains to be investigated. The present work showed that staurosporine significantly upregulated expression of BLK, while MSCs co-culture inhibited upregulation of BLK expression in a partially cell-contact dependent manner. It can be postulated that BLK might be invovled in apoptosis and inflammatory response of hepatocytes as BLK is reported to participate in pre-B-cell receptor-mediated NF-κB activation (Krappmann *et al.*, 2001), while activation of NF-κB is known to determine the balance between apoptosis and proliferation of hepatocytes in response to TNF-α during liver regeneration (Plümpe *et al.*, 2000).

5.4.5 Conclusions

In conclusion, MSCs that expressed no TNF-α themselves significantly decreased autocrine TNF-α activity of co-cultured hepatocytes in a cell contact dependent manner. HPc further potentiated the inhibitory effect of MSCs co-culture by an intracellular ROS dependent mechanism. Inhibited autocrine activity of TNF-α might be a negative regulating mechanism of MSCs co-culture. Heterotypic interaction between hepatocytes and MSCs increased TGF-β secretion in co-culture, and autocrine TGF-β activity of MSCs was required for the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. Cell contact and intracellular ROS dependent extracellular collagen deposit of MSCs also played an essential role in trophic and antiapoptotic effect of MSCs on co-cultured hepatocytes. The underlying molecular mechanisms included downregulation of caspase-9 and decreased *BAX*/*BCL-2* ratio, while the implication of BLK in hepatocyte biology and MSCs co-culture remains to be investigated.

CHAPTER 6 CONCLUDING DISCUSSION AND FUTURE WORK

6.1 Concluding Discussion

6.1.1 *Trophic and protective effects of MSCs on co-cultured hepatocytes*

In the present work, the well documented trophic effect of MSCs on co-cultured porcine (Gu *et al.*, 2009[a]; Gu *et al.*, 2009[a]; Gu *et al.*, 2009[c]) or rodent (Shi *et al.*, 2009) hepatocytes was reproduced in primary human hepatocytes. The trophic effect of MSCs also applied to steatotic and cryopreserved hepatocytes, both of which are frequently encountered and used in the practice of HCT. This trophic effect with respect to liver-specific metabolic function might result from improved hepatocyte survival, potentiated metabolism of surviving hepatocytes, or both. MSCs transplantation was reported to ameliorate hepatocyte apoptosis following ischaemia/reperfusion injury (Pan *et al.*, 2012) or CCl_4 exposure (Manuelpillai *et al.*, 2010). A novel contribution of the present work was the finding that MSCs significantly inhibited caspase-mediated spontaneous and chemically-induced apoptosis of co-cultured hepatocytes. Furthermore, MSCs co-culture reversed staurosporine-induced necrosis-to-apoptosis switch in death mode of hepatocytes. Supportive effect of MSCs for co-cultured epithelial cells is deduced to be the synergistic effect of soluble factors, ECM, and cell-to-cell crosstalk. In contrast to previous studies reporting a positive role of paracrine factors in the hepatotrophic effects of MSCs (Gu *et al.*, 2009[a]), the present work showed that soluble factors released from MSCs alone or MSCs co-cultured with hepatocytes contributed minimally to the trophic and protective effects of MSCs co-culture although co-culture might amplify paracrine activity of MSCs-derived soluble factors. This contradiction might be explained as species variation (human *versus* porcine) so that human primary hepatocytes became unresponsive to the stimuli with potential soluble factors. Conversely, this finding suggested that trophic and antiapoptotic effects of MSCs co-culture might result primarily from additive or synergistic effects of ECM and cell-to-cell interaction. Deprivation of ECM (Sérandour *et al.*, 2005) and cell-to-cell contact (Gómez-Aristizábal and Davies, 2012) has an adverse effect on hepatocyte proliferation and metabolism.

The present work also demonstrated that the extent of the hepatotrophic effect of co-culture remained similar among AT-, BM-, and UC-MSCs. Co-culture with any type of MSCs improved liver-specific metabolic function of hepatocytes to a significantly greater extent as compared to that with ADFs. Moreover, AT-MSCs even at a low seeding ratio to hepatocytes still exhibited a significant hepatotrophic effect comparable to those at a high seeding ratio. This finding suggested that MSCs contributed to hepatotrophic effect of co-culture by a stem cell-specific mechanism in addition to providing hepatocytes with an attachment matrix.

It will be worthwhile investigating the possibility of using autologous liver-derived MSCs for co-culture with hepatocytes. Liver-derived MSCs is a potential progenitor reservoir of hepatocytes through transdifferentiation in addition to a supportive cell population for hepatocytes (Najimi *et al.*, 2007). The advantages of using liver-derived MSCs are the synergistic effect of hepatocytes and

MSCs isolated from a single donor liver and potentially better cellular engraftment into the liver (Moreno *et al.*, 2012). The present work showed that co-culture with a small number of hepatic NPCs possibly containing liver MSCs significantly improved viability and liver-specific metabolism of hepatocytes as evidenced by the preliminary results. The major limitation of the experiments with hepatic NPCs was poor reproducibility and propagatability. Further effort should be made to produce a constant, reliable hepatic NPC line, and this cell population should be fractioned and characterised to identify the underlying stem cell subpopulation. Steatotic liver may be a suitable source for isolating liver NPCs including MSCs and hepatic progenitor cells (Tolosa *et al.*, 2011); however, use of steatotic liver-derived NPCs carries a potential profibrogenic risk as NF-κB activation in liver NPCs mediates inflammatory response and bridges steatohepatitis and liver fibrosis (Beraza *et al.*, 2008). It will be of great significance to investigate how co-culture with hepatocytes modulates the biological behaviour of MSCs. It was reported that co-culture with human liver cells drove differentiation of rat MSCs into hepatocyte-like cells in a spheroid architecture in the presence of HGF (Qihao *et al.*, 2007). Lange *et al.* (World J Gastroenterol, 2005[b]; World J Gastroenterol, 2006) detected hepatocytic differentiation of rat MSCs co-cultured with adult rat or foetal liver cells, as evidenced by expression of hepatocyte-specific markers, in the absence of chemically-defined culture medium. Hepatotrophic effect of MSCs co-culture may also partially result from hepatogenic transdifferentiation of MSCs especially after transplantation and engraftment into the liver.

6.1.2 *HPc potentiates hepatotrophic and antiapoptotic effects of MSCs co-culture by an intracellular ROS dependent mechanism*

The present work showed that HPc potentiated trophic and antiapoptotic effect of MSCs on hepatocytes as compared to NPc. Potentiative effect of HPc on MSCs themselves has been extensively investigated, and HPc-primed MSCs exhibit an enhanced supportive and protective effect on the host cells, such as hepatocytes (Yu *et al.*, 2013), cardiomyocytes (He *et al.*, 2009), and neural cells (Wei *et al.*, 2012). Potentiated paracrine activity is thought to be a major contributive factor of HPc-induced potentiative effect; however, the present work showed indirect noncontact co-culture with HPc-MSCs did not significantly improved cellular viability and metabolic function of hepatocytes. The probable explanation was that freshly isolated hepatocytes still remained unresponsive even in the presence of potentiated extrinsic cues due to mechanical and enzymatic stress from isolation. Conversely, the absence of potentiative effect in HPc indirect co-culture implied that HPc potentiated MSCs mainly by an mechanism involving ECM and/or cell-to-cell interplay.

NAC pretreatment diminished the increase of intracellular ROS activity in MSCs and reduced HPc-potentiated trophic and antiapoptotic effects of MSCs on co-cultured MSCs. This finding was consistent with that reported by De Barros *et al.* (2013), in which HPc counteracted ageing-related impairment of angiogenic potential in human AT-MSCs. The improved angiogenesis might be the

synergetic effect of MSCs transdifferentiation into endothelial cells, enhanced expression of proangiogenic and prosurvival factors, and oxidative stress. The major signal was determined to be intracellular ROS as NAC pretreatment reversed HPc-induced improvement. A second potential candidate factor responsible for HPc-induced potentiative effect is HIF, a family of transcription factors sensitive to oxygen tension in the microenvironment. Activation of HIF participates in the inhibitory effect of hypoxia on MSCs differentiation (Haque *et al.*, 2013) but improves angiogenesis (Hu *et al.*, 2008) and migration (Liu *et al.*, 2011) of MSCs. It will also be important to investigate what beneficial mechanisms, especially those invovled in ECM formation and cell-to-cell crosstalk are activated by oxygen-sensoring factors, such as ROS and HIF, and how these mechanisms potentiate trophic and protective effect of MSCs on co-cultured hepatocytes.

6.1.3 *Molecules underlying hepatotrophic effect of MSCs co-culture and potentiative effect of HPc induction*

The trophic and protective effects of MSCs on co-cultured hepatocytes are attributed to synergistic effects of soluble factors, ECM, and heterotypic cellular interaction. As a result, it is likely that HPc potentiated the hepatotrophic and antiapoptotic effects of MSCs by enhancing these mechanistic factors. TNF-α is thought to play an essential role in mediating hepatocyte apoptosis in the setting of inflammatory response (Zang *et al.*, 2000; Biburger and Tiegs, 2005). The present work showed that hepatocytes secreted a relatively high level of baseline TNF-α and neutralisation of baseline TNF-α reduced apoptosis and total death of hepatocytes. In addition, hepatocyte secretion of TNF-α was significantly inhibited by co-culture with MSCs and by HPc-MSCs to a significantly greater extent. This inhibitory effect of MSCs on co-cultured hepatocytes also depended on MSC-to-hepatocyte contact as evidenced by the minimal inhibitory effect seen in the indirect non-contact co-culture. Therefore, MSCs co-culture might suppress autocrine TNF-α activity of hepatocytes by a cell-to-cell contact dependent mechanism. The possibility that decreased autocrine TNF-α activity resulted in inhibition of hepatocyte apoptosis remains an open question as MSCs co-culture CM contained a low level of TNF-α but had no effect on hepatocyte apoptosis. It is possible that reduced autocrine TNF-α activity is a prerequisite rather than sufficient condition for antiapoptotic effect of MSCs co-culture. It remains to be investigated by which mechanism MSCs co-culture and HPc induction inhibited autocrine TNF-α activity of hepatocytes. A possibility is that TNF-α synergistically coordinates with other proapoptotic factors by a positive feedback mechanism in hepatocytes undergoing spontaneous apoptosis (Schlatter *et al.*, 2011). Interruption of this positive feedback will reduce hepatocyte apoptosis and consequently inhibit autocrine TNF-α activity.

TGF-β is known to be involved in liver fibrosis by promoting collagen deposition (Gabriel *et al.*, 2008); however, TGF-β positively regulates osteochondrogenesis of MSCs (Mehlhorn *et al.*, 2006). Contact of MSCs with T-lymphocytes also upregulates expression of IL-10 and TGF-β, both of which mediates T-cell tolerance (Nasef *et al.*, 2007). The present work showed that MSCs-to-hepatocytes contact and HPc increased TGF-β1 secretion in co-culture and by MSCs, respectively.

Neutralising autocrine activity of TGF-β1 in both NPc- and HPc-MSCs led to a significant reduction of MSCs co-culture antiapoptotic effect. As MSCs co-culture CM containing high-level TGF-β1 had no significant effect on hepatocyte apoptosis, enhanced TGF-β1 expression in co-culture and HPc-MSCs contributed to the antiapoptotic effect of MSCs co-culture by an MSC-autocrine rather hepatocyte-paracrine mechanism. This contact co-culture induced enhancement might result from activation of latent TGF-β1 rather than upregulation of TGF-β1 transcriptional and translational expression (Chia *et al.*, 2005). It is an open question how activation of autocrine TGF-β1 in MSCs inhibits apoptosis of co-cultured hepatocytes. Mediation of ECM and cell-to-cell interplay may be the candidate mechanism. TGF-β1 is reported to modulate MSCs attachment to collagen type I by an integrin-mediated mechanism (Warstat *et al.*, 2010).

The primary effect of collagen on hepatocytes is provision of an attachment matrix and also regulation of hepatocyte cell cycle progression and differentiation (Hansen *et al.*, 2006). Under physiological conditions, liver collagen is mainly produced by NPCs rather than hepatocytes; however, collagen deprivation will be detrimental for hepatocytes cultured *in vitro*. The present work demonstrated that extracellular collagen, the active form of collagen, was mainly deposited by MSCs rather than hepatocytes. Cell contact and HPc also enhanced extracellular collagen deposit of MSCs co-culture and MSCs, respectively. Antagonisation experiments showed that inhibition of extracellular collagen deposit by MSCs diminished the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. Conventional hepatocyte mono-culture only allows hepatocyte monolayer collagen attachment, while MSCs co-culture supports hepatocytes in a three-dimensional architecture (Gu *et al.*, 2009[b]). Three-dimensional hepatocyte culture in sandwich collagen gel is thought to mimic the microenvironment and shows a supportive effect superior to conventional monolayer collagen architecture (Wang *et al.*, 2004). Collagen may also regulate responsiveness of hepatocytes to soluble factors and intercellular interaction (Berthiaume *et al.*, 1996). It will be academically significant to investigate what subtype(s) of collagen is increased in MSCs co-culture and after HPc induction as biological activities of collagen vary significantly among subtypes. It is also interesting to delineate by which mechanism, such as modification of collagen topography, shift of collagen isoforms, and amplification of soluble factors and cellular interplay, enhanced collagen deposition results in the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction.

Apoptotic death of hepatocytes is a biological event finely modulated by a complex network of proapoptotic and antiapoptotic factors. The results of Chapter 3 showed that MSCs co-culture significantly inhibited caspase-mediated CK18 cleavage, an early-stage event in the process of cellular apoptosis, as evidenced by downregulated expression of caspase-9 in MSCs co-culture. This finding suggested that MSCs co-culture mainly inhibited initiation of hepatocyte apoptosis mediated by the mitochondrial pathway. Additionally, decreased *BAX*/*BCL-2* ratio indicated that MSCs co-culture suppressed the death receptor-associated apoptosis pathway. The present work also demonstrated for the first time that MSCs co-culture downregulated expression of *BLK*, biological activities of which are rarely reported in hepatocytes, at the transcriptional level by a cell contact,

intracellular ROS-dependent mechanism. It remains unknown how the potential contributive factors of MSCs co-culture, such as hepatocyte-autocrine TNF-α, MSC-autocrine TGF-β1, and extracellular collagen, modulate the balance between proapoptosis and antiapoptosis in hepatocytes by a cell contact, intracellular ROS-dependent mechanism. It is impossible for a single factor or mechanism to fully explain the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction. It will also be informative to investigate the reciprocal interaction between hepatocytes and MSCs using genomic microarray techniques.

6.2 Conclusions

In conclusion, MSCs have trophic, antiapoptotic, prosurvival, and protective effects on co-cultured hepatocytes. These favourable effects can also be reproduced in functionally impaired steatotic and cryopreserved hepatocytes. The paracrine contribution of soluble factors to hepatotrophic effect of MSCs co-culture that has been well documented in animal-derived hepatocytes could not be reproduced in the present work possibly due to the unresponsiveness of human hepatocytes to extrinsic cues in the absence of ECM and intercellular crosstalk. HPc significantly potentiated the hepatotrophic effects of MSCs co-culture by an intracellular ROS-dependent mechanism, while this potentiative effect also depended on heterotypic cellular interaction as evidenced by the minimal effect of HPc indirect noncontact co-culture. Potential contributive factors to the hepatotrophic effects of MSCs co-culture and potentiative effect of HPc induction were decreased hepatocyte autocrine activity of TNF-α, increased MSC autocrine activity of TGF-β1, and enhanced extracellular collagen deposit by MSCs, as evidenced by the reversal effects of neutralisation or inhibition experiments. These potential contributive factors may synergistically switch the balance from proapoptosis to antiapoptosis in hepatocytes co-cultured with MSCs.

With respect to the bench-to-bedside translation of the present work, co-transplantation with MSCs is expected to improve therapeutic efficacy of current HCT facing two major hurdles, limited donor tissue availability and marginal cell quality. Co-transplantation of MSCs may offer additional benefits, such as transdifferentiation into hepatocytes, repair of the injured host hepatocytes, improvement of neovascularisation *in vivo*, and modulation of immune response. HPc may also potentiate these therapeutic effects of MSCs *in vivo* like hepatotrophic and antiapoptotic effects of MSCs co-culture *in vitro* observed in the present work. However, the effectiveness and safety of MSCs co-transplantation and HPc modification needs to be validated in preclinical liver failure models. A major safety concern arising from incorporation of MSCs to HCT is that MSCs carry a potential risk of fibrogenesis especially in the profibrotic environment.

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LIST OF PUBLICATIONS

- ***Peer-reviewed article***

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- ***Book chapter***

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- ***Conference paper***

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  Qin HH, Mitry RR, Dhawan A, Hughes RD. Hypoxia-preconditioned mesenchymal stem cells suppress caspase-specific apoptosis and improve liver-specific metabolic function of co-cultured human hepatocytes. *The 46th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition*, 8th–11th May, 2012, London, United Kingdom (Prize-winning oral presentation).

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